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JCS946 U.S. PRO

**TRANSMITTAL OF  
UTILITY  
APPLICATION  
UNDER 37  
C.F.R. §1.53**

Attorney Docket No.	24736-2033
First named inventor	Andreas Braun
Express mail label #	EL406143913US
Date of mailing	October 13, 2000

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**Application Elements**

1. ☒ Fee Transmittal Form
2. ☒ Specification containing 135 pages (including claims and Abstract), a Sequence Listing (110 pages).
  - a. Title: **METHODS FOR GENERATING DATABASES AND DATABASES FOR IDENTIFYING POLYMORPHIC GENETIC MARKERS**
  - b. Number of claims: 100
3. ☒ 49 sheets of drawings with 58 Figures
4. ☐ Declaration
5. ☒ Sequence Listing
  - ☒ Paper copy (identical to computer copy)
  - ☒ Computer readable copy
  - ☐ Verified statement

**Accompanying Application Papers**

6. ☐ Assignments
7. ☒ Small Entity Status is claimed
8. ☐ Preliminary Amendment
9. ☒ Return Receipt Postcard

**SIGNATURE OF ATTORNEY/AGENT**

HELLER EHRMAN WHITE &amp; McAULIFFE LLP

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Registration Number: 33,779

[X] Benefit of priority under 35 U.S.C. §119(e) claimed to U.S. provisional application Serial Nos. 60/217,658, 60/159,176 and 60/217,251. Benefit of priority under 35 U.S.C. §120 is also claimed. This application is a continuation-in-part of U.S. application Serial No. 09/663,938, filed September 19, 2000. The application and provisional applications are incorporated by reference in their entirety

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09687493-101300

**FEE TRANSMITTAL  
ACCOMPANYING UTILITY  
APPLICATION UNDER  
37 C.F.R. §1.53**


Attorney Docket No.	24736-2033
First named inventor	Andreas Braun
Express mail label #	EL406143913US
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**FEE CALCULATION FOR CLAIMS AS AMENDED**

a)	Basic Fee		\$ 710.00
b)	Independent Claims $\frac{15}{15} - 3 = \frac{12}{12} \times \$ 80.00$		\$ 960.00
c)	Total Claims $\frac{100}{100} - 20 = \frac{80}{80} \times \$ 18.00$		\$1440.00
d)	Fee for Multiple Dependent Claims - \$270.00		\$ 0.00
	<b>TOTAL FILING FEE</b>		<b>\$3110.00</b>

- [X] Status as Small Entity is claimed reducing Fee by one-half to \$1555.00
- [X] A check in the amount of \$1550.00 to cover the fee for filing the application.
- [ ] Charge \$ .00 to Deposit Account No. 50-1213
- [X] The Commissioner is hereby authorized to charge any fees, including the filing fee and excess claims fee, that may be required in this application under 37 C.F.R. §§ 1.16-1.17 during its entire pendency, or credit any overpayment, to Deposit Account No. 50-1213. If proper payment is not enclosed, such as a check in the wrong amount, unsigned, post-dated, otherwise improper or informal, or absent, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 50-1213 during the entire pendency of this application. This sheet is filed in duplicate.

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09687483-101300

**METHODS FOR GENERATING DATABASES AND DATABASES FOR  
IDENTIFYING POLYMORPHIC GENETIC MARKERS**

**RELATED APPLICATIONS**

Benefit of priority under 35 U.S.C. §119(e) to the following

- 5 provisional applications is claimed herein:
- U.S. provisional application Serial No. 60/217,658 to Andreas Braun, Hubert Koster; Dirk Van den Boom, filed July 10, entitled "METHODS FOR GENERATING DATABASES AND DATABASES FOR IDENTIFYING POLYMORPHIC GENETIC MARKERS"; U.S. provisional application Serial
- 10 No. 60/159,176 to Andreas Braun, Hubert Koster, Dirk Van den Boom, filed October 13, 1999, entitled "METHODS FOR GENERATING DATABASES AND DATABASES FOR IDENTIFYING POLYMORPHIC GENETIC MARKERS"; U.S. provisional application Serial No. 60/217,251, filed July 10, 2000, to Andreas Braun, entitled "POLYMORPHIC KINASE
- 15 ANCHOR PROTEIN GENE SEQUENCES, POLYMORPHIC KINASE ANCHOR PROTEINS AND METHODS OF DETECTING POLYMORPHIC KINASE ANCHOR PROTEINS AND NUCLEIC ACIDS ENCODING THE SAME". This application is also a continuation-in-part of U.S. application Serial No. 09/663,968, to Ping Yip, filed September 19, 2000, entitled "METHOD
- 20 AND DEVICE FOR IDENTIFYING A BIOLOGICAL SAMPLE."

The above-noted applications and provisional applications are incorporated by reference in their entirety.

**FIELD OF THE INVENTION**

- Process and methods for creating a database of genomic samples
- 25 from healthy human donors. Methods that use the database to identify and correlate with polymorphic genetic markers and other markers with diseases and conditions are provided.

**BACKGROUND**

- Diseases in all organisms have a genetic component, whether
- 30 inherited or resulting from the body's response to environmental stresses, such as viruses and toxins. The ultimate goal of ongoing genomic

research is to use this information to develop new ways to identify, treat and potentially cure these diseases. The first step has been to screen disease tissue and identify genomic changes at the level of individual samples. The identification of these "disease" markers has then fueled

5 the development and commercialization of diagnostic tests that detect these errant genes or polymorphisms. With the increasing numbers of genetic markers, including single nucleotide polymorphisms (SNPs), microsatellites, tandem repeats, newly mapped introns and exons, the challenge to the medical and pharmaceutical communities is to identify

10 genotypes which not only identify the disease but also follow the progression of the disease and are predictive of an organism's response to treatment.

Currently the pharmaceutical and biotechnology industries find a disease and then attempt to determine the genomic basis for the disease.

15 This approach is time consuming and expensive and in many cases involves the investigator guessing as to what pathways might be involved in the disease.

### **Genomics**

Presently the two main strategies employed in analyzing the

20 available genomic information are the technology driven reverse genetics brute force strategy and the knowledge-based pathway oriented forward genetics strategy. The brute force approach yields large databases of sequence information but little information about the medical or other uses of the sequence information. Hence this strategy yields intangible

25 products of questionable value. The knowledge-based strategy yields small databases that contain a lot of information about medical uses of particular DNA sequences and other products in the pathway and yield tangible products with a high value.

### Polymorphisms

Polymorphisms have been known since 1901 with the identification of blood types. In the 1950's they were identified on the level of proteins using large population genetic studies. In the 1980's and 1990's many of the known protein polymorphisms were correlated with genetic loci on genomic DNA. For example, the gene dose of the apolipoprotein E type 4 allele was correlated with the risk of Alzheimer's disease in late onset families (see, *e.g.*, Corder *et al.* (1993) *Science* 261: 921-923; mutation in blood coagulation factor V was associated with resistance to activated protein C (see, *e.g.*, Bertina *et al.* (1994) *Nature* 369:64-67); resistance to HIV-1 infection has been shown in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene (see, *e.g.*, Samson *et al.* (1996) *Nature* 382:722-725); and a hypermutable tract in antigen presenting cells (APC, such as macrophages), has been identified in familial colorectal cancer in individuals of Ashkenazi Jewish background (see, *e.g.*, Laken *et al.* (1997) *Nature Genet.* 17:79-83). There may be more than three million polymorphic sites in the human genome. Many have been identified, but not yet characterized or mapped or associated with a marker.

### Single nucleotide polymorphisms (SNPs)

Much of the focus of genomics has been in the identification of SNPs, which are important for a variety of reasons. They allow indirect testing (association of haplotypes) and direct testing (functional variants). They are the most abundant and stable genetic markers. Common diseases are best explained by common genetic alterations, and the natural variation in the human population aids in understanding disease, therapy and environmental interactions.

Currently, the only available method to identify SNPs in DNA is by sequencing, which is expensive, difficult and laborious. Furthermore, once a SNP is discovered it must be validated to determine if it is a real

- polymorphism and not a sequencing error. Also, discovered SNPs must then be evaluated to determine if they are associated with a particular phenotype. Thus, there is a need to develop new paradigms for identifying the genomic basis for disease and markers thereof. Therefore,
- 5 it is an object herein to provide methods for identifying the genomic basis of disease and markers thereof.

### SUMMARY

- Databases and methods using the databases are provided herein. The databases comprise sets of parameters associated with subjects in
- 10 populations selected only on the basis of being healthy (*i.e.*, where the subjects are mammals, such as humans, they are selected based upon apparent health and no detectable infections). The databases can be sorted based upon one or more of the selected parameters.
- The databases are preferably relational databases, in which an
- 15 index that represents each subject serves to relate parameters, which are the data, such as age, ethnicity, sex, medical history, etc. and ultimately genotypic information, that was inputted into and stored in the database. The database can then be sorted according to these parameters. Initially, the parameter information is obtained from a questionnaire answered by
- 20 each subject from whom a body tissue or body fluid sample is obtained. As additional information about each sample is obtained, this information can be entered into the database and can serve as a sorting parameter.

- The databases obtained from healthy individuals have numerous uses, such as correlating known polymorphisms with a phenotype or
- 25 disease. The databases can be used to identify alleles that are deleterious, that are beneficial, and that are correlated with diseases.

For purposes herein, genotypic information can be obtained by any method known to those of skill in the art, but is preferably obtained using mass spectrometry.

Also provided herein, is a new use for existing databases of subjects and genotypic and other parameters, such as age, ethnicity, race, and gender. Any database can be sorted according to the methods herein and alleles that exhibit statistically significant correlations with any

5 of the sorting parameters can be identified. It is noted, however, is noted, that the databases provided herein and randomly selected databases will perform better in these methods, since disease-based databases suffer numerous limitations, including their relatively small size, the homogeneity of the selected disease population, and the masking

10 effect of the polymorphism associated with the markers for which the database was selected. Hence, the healthy database provided herein, provides advantages not heretofore recognized or exploited. However, the methods provided herein can be used with a selected database, including disease-based databases, with or without sorting for the

15 discovery and correlation of polymorphisms. In addition, the databases provided herein represent a greater genetic diversity than the unselected databases typically utilized for the discovery of polymorphisms and thus allow for the enhanced discovery and correlation of polymorphisms.

The databases provided herein can be used for taking an identified

20 polymorphism, and ascertaining whether it changes in frequency when the data is sorted according to a selected parameter.

One use of these methods is correlating a selected marker with a particular parameter by following the occurrence of known genetic markers and then, having made this correlation, determining or identifying

25 correlations with diseases. Examples of this use are p53 and Lipoprotein Lipase polymorphism. As exemplified herein, known markers are shown

to have particular correlation with certain groups, such as a particular ethnicity or race or one sex. Such correlations will then permit development of better diagnostic tests and treatment regimens.

These methods are valuable for identifying one or more genetic  
5 markers whose frequency changes within the population as a function of age, ethnic group, sex or some other criteria. This can allow the identification of previously unknown polymorphisms and ultimately a gene or pathway involved in the onset and progression of disease.

The databases and methods provided herein permit, among other  
10 things, identification of components, particularly key components, of a disease process by understanding its genetic underpinnings and also permit an understanding of processes, such as individual drug responses. The databases and methods provided herein also can be used in methods involving elucidation of pathological pathways, in developing new  
15 diagnostic assays, identifying new potential drug targets, and in identifying new drug candidates.

The methods and databases can be used with experimental procedures, including, but are not limited to, *in silico* SNP identification, *in vitro* SNP identification/verification, genetic profiling of large populations,  
20 and in biostatistical analyses and interpretations.

Also provided herein, are combinations that contain a database provided herein and a biological sample from a subject in the database, and preferably biological samples from all subjects or a plurality of subjects in the database. Collections of the tissue and body fluid samples  
25 are also provided.

Also, provided herein, are methods for determining a genetic marker that correlates with age, comprising identifying a polymorphism and determining the frequency of the polymorphism with increasing age in a healthy population.

Further provided herein are methods for determining whether a genetic marker correlates with susceptibility to morbidity, early mortality, or morbidity and early mortality, comprising identifying a polymorphism and determining the frequency of the polymorphism with increasing age in  
5 a healthy population.

Any of the methods herein described can be used out in a multiplex format.

Also provided are an apparatus and process for accurately identifying genetic information. It is another object of the herein that  
10 genetic information be extracted from genetic data in a highly automated manner. Therefore, to overcome the deficiencies in the known conventional systems, a method and apparatus for identifying a biological sample is proposed.

Briefly, the method and system for identifying a biological sample  
15 generates a data set indicative of the composition of the biological sample. In a particular example, the data set is DNA spectrometry data received from a mass spectrometer. The data set is denoised, and a baseline is deleted. Since possible compositions of the biological sample may be known, expected peak areas may be determined. Using the  
20 expected peak areas, a residual baseline is generated to further correct the data set. Probable peaks are then identifiable in the corrected data set, which are used to identify the composition of the biological sample. In a disclosed example, statistical methods are employed to determine the probability that a probable peak is an actual peak, not an actual peak, or  
25 that the data too inconclusive to call.

Advantageously, the method and system for identifying a biological sample accurately makes composition calls in a highly automated manner. In such a manner, complete SNP profile information, for example, may be collected efficiently. More importantly, the collected data is analyzed  
30 with highly accurate results. For example, when a particular composition

is called, the result may be relied upon with great confidence. Such confidence is provided by the robust computational process employed .

#### DESCRIPTION OF THE DRAWINGS

Figure 1 depicts an exemplary sample bank. Panel 1 shows the  
5 samples as a function of sex and ethnicity. Panel 2 shows the  
caucasians as a function of age. Panel 3 shows the Hispanics as a  
function of age.

Figures 2A and 2C show an age- and sex-distribution of the 291S  
allele of the lipoprotein lipase gene in which a total of 436 males and 589  
10 females were investigated. Figure 2B shows an age distribution for the  
436 males.

Figure 3 is an exemplary questionnaire for population-based sample  
banking.

Figure 4 depicts processing and tracking of blood sample  
15 components.

Figure 5 depicts the allelic frequency of "sick" alleles and "healthy"  
alleles as a function of age. It is noted that the relative frequency of  
healthy alleles increases in a population with increasing age.

Figure 6 depicts the age-dependent distribution of ApoE genotypes  
20 (see, Schächter *et al.* (1994) *Nature Genetics* 6:29-32).

Figure 7A-D depicts age-related and genotype frequency of the p53  
(tumor suppressor) codon 72 among the caucasian population in the  
database. \*R72 and \*P72 represent the frequency of the allele in the  
database population. R72, R72P, and P72 represent the genotypes of the  
25 individuals in the population. The frequency of the homozygous P72  
allele drops from 6.7% to 3.7% with age.

Figure 8 depicts the allele and genotype frequencies of the p21  
S31R allele as a function of age.

Figure 9 depicts the frequency of the FVII Allele 353Q in pooled  
30 versus individual samples.

Figure 10 depicts the frequency of the CETP (cholesterol ester transfer protein) allele in pooled versus individual samples

Figure 11 depicts the frequency of the plasminogen activator inhibitor-1 (PAI-1) 5G in pooled versus individual samples

5      Figure 12 shows mass spectra of the samples and the ethnic diversity of the PAI-1 alleles.

Figure 13 shows mass spectra of the samples and the ethnic diversity of the CETP 405 alleles.

10      Figure 14 shows mass spectra of the samples and the ethnic diversity of the Factor VII 353 alleles.

Figure 15 shows ethnic diversity of PAI-1, CETP and Factor VII using the pooled DNA samples.

Figure 16 shows the p53-Rb pathway and the relationships among the various factors in the pathway.

15      Figure 17, which is a block diagram of a computer constructed to provide and process the databases described herein, depicts a typical computer system for storing and sorting the databases provided herein and practicing the methods provided herein.

20      Figure 18 is a flow diagram that illustrates the processing steps performed using the computer illustrated in Figure 17, to maintain and provide access to the databases for identifying polymorphic genetic markers.

25      Figure 19 is a histogram showing the allele and genotype distribution in the age and sex stratified Caucasian population for the AKAP10-1 locus. Bright green bars show frequencies in individuals younger than 40 years. Dark green bars show frequencies in individuals older than 60 years.

30      Figure 20 is a histogram showing the allele and genotype distribution in the age and sex stratified Caucasian population for the AKAP10-5 locus. Bright green bars show frequencies in individuals

younger than 40 years; dark green bars show frequencies in individuals older than 60 years.

Figure 21 is a histogram showing the allele and genotype distribution in the age and sex stratified Caucasian population for the h-msrA locus. Genotype difference between male age groups is significant. Bright green bars show frequencies in individuals younger than 40 years. Dark green bars show frequencies in individuals older than 60 years.

Figure 22A-D is a sample data collection questionnaire used for the healthy database.

10 Figure 23 is a flowchart showing processing performed by the computing device of Figure 24 when performing genotyping of sense strands and antisense strands from assay fragments.

Figure 24 is a block diagram showing a system in accordance with the present invention;

15 Figure 25 is a flowchart of a method of identifying a biological sample in accordance with the present invention;

Figure 26 is a graphical representation of data from a mass spectrometer;

20 Figure 27 is a diagram of wavelet transformation of mass spectrometry data;

Figure 28 is a graphical representation of wavelet stage 0 hi data;

Figure 29 is a graphical representation of stage 0 noise profile;

Figure 30 is a graphical representation of generating stage noise standard deviations;

25 Figure 31 is a graphical representation of applying a threshold to data stages;

Figure 32 is a graphical representation of a sparse data set;

Figure 33 is a formula for signal shifting;

30 Figure 34 is a graphical representation of a wavelet transformation of a denoised and shifted signal;

Figure 35 is a graphical representation of a denoised and shifted signal;

Figure 36 is a graphical representation of removing peak sections;

Figure 37 is a graphical representation of generating a peak free  
5 signal ;

Figure 38 is a block diagram of a method of generating a baseline correction;

Figure 39 is a graphical representation of a baseline and signal;

Figure 40 is a graphical representation of a signal with baseline  
10 removed;

Figure 41 is a table showing compressed data;

Figure 42 is a flowchart of method for compressing data;

Figure 43 is a graphical representation of mass shifting;

Figure 44 is a graphical representation of determining peak width;

Figure 45 is a graphical representation of removing peaks;  
15

Figure 46 is a graphical representation of a signal with peaks removed;

Figure 47 is a graphical representation of a residual baseline;

Figure 48 is a graphical representation of a signal with residual  
20 baseline removed;

Figure 49 is a graphical representation of determining peak height;

Figure 50 is a graphical representation of determining signal-to-noise for each peak;

Figure 51 is a graphical representation of determining a residual  
25 error for each peak;

Figure 52 is a graphical representation of peak probabilities;

Figure 53 is a graphical representation of applying an allelic ratio to peak probability;

Figure 54 is a graphical representation of determining peak  
30 probability

Figure 55 is a graphical representation of calling a genotype;

Figure 56 is a flowchart showing a statistical procedure for calling a genotype;

Figure 57 is a flowchart showing processing performed by the  
5 computing device of Figure 1 when performing standardless genotyping;  
and

Figure 58 is graphical representation of applying an allelic ratio to peak probability for standardless genotype processing.

#### **DETAILED DESCRIPTION**

##### **10 Definitions**

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences  
15 from GenBank and other databases referred to herein throughout the disclosure are incorporated by reference in their entirety.

As used herein, a biopolymer includes, but is not limited to, nucleic acid, proteins, polysaccharides, lipids and other macromolecules. Nucleic acids include DNA, RNA, and fragments thereof. Nucleic acids may be  
20 derived from genomic DNA, RNA, mitochondrial nucleic acid, chloroplast nucleic acid and other organelles with separate genetic material.

As used herein, morbidity refers to conditions, such as diseases or disorders, that compromise the health and well-being of an organism, such as an animal. Morbidity susceptibility or morbidity-associated genes  
25 are genes that, when altered, for example, by a variation in nucleotide sequence, facilitate the expression of a specific disease clinical phenotype. Thus, morbidity susceptibility genes have the potential, upon alteration, of increasing the likelihood or general risk that an organism will develop a specific disease.

As used herein, mortality refers to the statistical likelihood that an organism, particularly an animal, will not survive a full predicted lifespan. Hence, a trait or a marker, such as a polymorphism, associated with increased mortality is observed at a lower frequency in older than younger segments of a population.

As used herein, a polymorphism, e.g. genetic variation, refers to a variation in the sequence of a gene in the genome amongst a population, such as allelic variations and other variations that arise or are observed. Thus, a polymorphism refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. These differences can occur in coding and non-coding portions of the genome, and can be manifested or detected as differences in nucleic acid sequences, gene expression, including, for example transcription, processing, translation, transport, protein processing, trafficking, DNA synthesis, expressed proteins, other gene products or products of biochemical pathways or in post-translational modifications and any other differences manifested amongst members of a population. A single nucleotide polymorphism (SNP) refers to a polymorphism that arises as the result of a single base change, such as an insertion, deletion or change in a base.

A polymorphic marker or site is the locus at which divergence occurs. Such site may be as small as one base pair (an SNP). Polymorphic markers include, but are not limited to, restriction fragment length polymorphisms, variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats and other repeating patterns, simple sequence repeats and insertional elements, such as Alu. Polymorphic forms also are manifested as different mendelian alleles for a gene. Polymorphisms may be observed by differences in proteins, protein modifications, RNA expression modification, DNA and RNA methylation,

regulatory factors that alter gene expression and DNA replication, and any other manifestation of alterations in genomic nucleic acid or organelle nucleic acids.

As used herein, a healthy population, refers to a population of  
5 organisms, including but are not limited to, animals, bacteria, viruses, parasites, plants, eubacteria, and others, that are disease free. The concept of disease-free is a function of the selected organism. For example, for mammals it refers to a subject not manifesting any disease state. Practically a healthy subject, when human, is defined as human  
10 donor who passes blood bank criteria to donate blood for eventual use in the general population. These criteria are as follows: free of detectable viral, bacterial, mycoplasma, and parasitic infections; not anemic; and then further selected based upon a questionnaire regarding history (see Figure 3). Thus, a healthy population represents an unbiased population  
15 of sufficient health to donate blood according to blood bank criteria, and not further selected for any disease state. Typically such individuals are not taking any medications. For plants, for example, it is a plant population that does not manifest diseases pathology associated with plants. For bacteria it is a bacterial population replicating without  
20 environmental stress, such as selective agents, heat and other pathogens.

As used herein, a healthy database (or healthy patient database) refers to a database of profiles of subjects that have not been pre-selected for any particular disease. Hence, the subjects that serve as the source of data for the database are selected, according to predetermined  
25 criteria, to be healthy. In contrast to other such databases that have been pre-selected for subjects with a particular disease or other characteristic, the subjects for the database provided herein are not so-selected. Also, if the subjects do manifest a disease or other condition, any polymorphism discovered or characterized should be related to an  
30 independent disease or condition. In a preferred embodiment, where the

subjects are human, a healthy subject manifests no disease symptoms and meets criteria, such as those set by blood banks for blood donors.

Thus, the subjects for the database are a population of any organism, including, but are not limited to, animals, plants, bacteria,  
5 viruses, parasites and any other organism or entity that has nucleic acid. Among preferred subjects are mammals, preferably, although not necessarily, humans. Such a database can capture the diversity of the a population, thus providing for discovery of rare polymorphisms.

As used herein, a profile refers to information relating to, but not  
10 limited to and not necessarily including all of, age, sex, ethnicity, disease history, family history, phenotypic characteristics, such as height and weight and other relevant parameters. A sample collect information form is shown in Figure 22, which illustrates profile intent.

As used herein, a disease state is a condition or abnormality or  
15 disorder that may be inherited or result from environmental stresses, such as toxins, bacterial, fungal and viral infections.

As used herein, set of non-selected subjects means that the subjects have not been pre-selected to share a common disease or other characteristic. They can be selected to be healthy as defined herein.

As used herein, a phenotype refers to a set of parameters that  
20 includes any distinguishable trait of an organism. A phenotype can be physical traits and can be, in instances in which the subject is an animal, a mental trait, such as emotional traits. Some phenotypes can be determined by observation elicited by questionnaires (see, *e.g.*, Figures 3  
25 and 22) or by referring to prior medical and other records. For purposes herein, a phenotype is a parameter around which the database can be sorted.

As used herein, a parameter is any input data that will serve as a basis for sorting the database. These parameters will include phenotypic  
30 traits, medical histories, family histories and any other such information

elicited from a subject or observed about the subject. A parameter may describe the subject, some historical or current environmental or social influence experienced by the subject, or a condition or environmental influence on someone related to the subject. Paramaters include, but are  
 5 not limited to, any of those described herein, and known to those of skill in the art.

As used herein, haplotype refers referes to two or polymorphism located on a single DNA strand. Hence, haplotyping refers to identification of two or more polymorphisms on a single DNA strand. Haplotypes can  
 10 be indicative of a phenotype. For some disorders a single polymorphism may suffice to indicate a trait; for others a plurality (i.e., a haplotype) may be needed. Haplotyping can be performed by isolating nucleic acid and separating the strands. In addition, when using enzymes such a certain nucleases, that produce, different size fragments from each strand, strand  
 15 separation is not needed for haplotyping.

As used herein, used herein, pattern with reference to a mass spectrum or mass spectrometric analyses, refers to a characteristic distribution and number of signals (such peaks or digital representations thereof).

20 As used herein, signal in the context of a mass spectrum and analysis thereof refers to the output data, which the number or relative number of molecules having a particular mass. Signals include "peaks" and digital representations thereof.

As used herein, adaptor, when used with reference to haplotyping  
 25 use Fen ligase, refers to a nucleic acid that specifically hybridizes to a polymorphism of ininterest. An adaptor can be partially double-stranded. An adaptor complex is formed when an adaptor hybridizes to its target.

As used herein, a target nucleic acid refers to any nucleic acid of interest in a sample. It can contain one or more nucleotides.

As used herein, standardless analysis refers to a determination based upon an internal standard. For example, the frequency of a polymorphism can be determined herein by comparing signals within a single mass spectrum.

- 5       As used herein, amplifying refers to means for increasing the amount of a biopolymer, especially nucleic acids. Based on the 5' and 3' primers that are chosen, amplification also serves to restrict and define the region of the genome which is subject to analysis. Amplification can be by any means known to those skilled in the art, including use of the
- 10       polymerase chain reaction (PCR) etc. Amplification, e.g., PCR must be done quantitatively when the frequency of polymorphism is required to be determined.

As used herein, cleaving refers to non-specific and specific fragmentation of a biopolymer.

- 15       As used herein, multiplexing refers to the simultaneous detection of more than one polymorphism. Methods for performing multiplexed reactions, particularly in conjunction with mass spectrometry are known (see, e.g., U.S. Patent Nos. 6,043,031, 5,547,835 and International PCT application No. WO 97/37041).

- 20       As used herein, reference to mass spectrometry encompasses any suitable mass spectrometric format known to those of skill in the art. Such formats include, but are not limited to, Matrix-Assisted Laser Desorption/Ionization, Time-of-Flight (MALDI-TOF), Electrospray (ES), IR-MALDI (see, e.g., published International PCT application No.99/57318 and U.S. Patent No. 5,118,937), Ion Cyclotron Resonance (ICR), Fourier Transform and combinations thereof. MALDI, particular UV and IR, are among the preferred formats.
- 25

As used herein, mass spectrum refers to the presentation of data obtained from analyzing a biopolymer or fragment thereof by mass spectrometry either graphically or encoded numerically.

As used herein, a blood component is a component that is  
5 separated from blood and includes, but is not limited to red blood cells and platelets, blood clotting factors, plasma, enzymes, plasminogen, immunoglobulins. A cellular blood component is a component of blood, such as a red blood cell, that is a cell. A blood protein is a protein that is normally found in blood. Examples of such proteins are blood factors VII  
10 and VIII. Such proteins and components are well-known to those of skill in the art.

As used herein, plasma can be prepared by any method known to those of skill in the art. For example, it can be prepared by centrifuging blood at a force that pellets the red cells and forms an interface between  
15 the red cells and the buffy coat, which contains leukocytes, above which is the plasma. For example, typical platelet concentrates contain at least about 10% plasma.

Blood may be separated into its components, including, but not limited to, plasma, platelets and red blood cells by any method known to  
20 those of skill in the art. For example, blood can be centrifuged for a sufficient time and at a sufficient acceleration to form a pellet containing the red blood cells. Leukocytes collect primarily at the interface of the pellet and supernatant in the buffy coat region. The supernatant, which contains plasma, platelets, and other blood components, may then be  
25 removed and centrifuged at a higher acceleration, whereby the platelets pellet.

As used herein, p53 is a cell cycle control protein that assesses DNA damage and acts as a transcription factor regulation gene which control cell growth, DNA repair and apoptosis. The p53 mutations have been found in a wide variety of different cancers, including all of the  
5 different types of leukemia, with varying frequency. The loss of normal p53 functions results in genomic instability and uncontrolled growth of the host cell.

As used herein, p21 is a cyclin-dependent kinase inhibitor, associated with G1 phase arrest of normal cells. Expression triggers  
10 apoptosis or programmed cell death and has been associated with Wilms' tumor, a pediatric kidney cancer.

As used herein, Factor VII is a serine protease involved the extrinsic blood coagulation cascade. This factor is activated by thrombin and works with tissue factor (Factor III) in the processing of Factor X to  
15 Factor Xa. Evidence has supported an association between polymorphisms in the gene and increase Factor VII activity which can result in an elevated risk of ischemic cardiovascular disease including myocardial infarction.

As used herein, a relational database stores information in a form  
20 representative of matrices, such as two-dimensional tables, including rows and columns of data, or higher dimensional matrices. For example, in one embodiment, the relational database has separate tables each with a parameter. The tables are linked with a record number, which also acts as an index. The database can be searched or sorted by using data in the  
25 tables and is stored in any suitable storage medium, such as floppy disk, CD rom disk, hard drive or other suitable medium.

As used herein, a bar codes refers any array of optically readable marks of any desired size and shape that are arranged in a reference context or frame of, preferably, although not necessarily, one or more  
30 columns and one or more rows. For purposes herein, the bar code refers

to any symbology, not necessary "bar" but may include dots, characters or any symbol or symbols.

- As used herein, symbology refers to an identifier code or symbol, such as a bar code, that is linked to a sample. The index will reference
- 5 each such symbology. The symbology is any code known or designed by the user. The symbols are associated with information stored in the database. For example, each sample can be uniquely identified with an encoded symbology. The parameters, such as the answers to the questions and subsequent genotypic and other information obtained upon
- 10 analysis of the samples is included in the database and associated with the symbology. The database is stored on any suitable recording medium, such as a hard drive, a floppy disk, a tape, a CD ROM, a DVD disk and any other suitable medium.

#### **DATABASES**

- 15 Human genotyping is currently dependent on collaborations with hospitals, tissues banks and research institutions that provide samples of disease tissue. This approach is based on the concept that the onset and/or progression of diseases can be correlated with the presence of a polymorphisms or other genetic markers. This approach does not
- 20 consider that disease correlated with the presence of specific markers and the absence of specific markers. It is shown herein that identification and scoring of the appearance and disappearance of markers is possible only if these markers are measured in the background of healthy subjects where the onset of disease does not mask the change in polymorphism
- 25 occurrence. Databases of information from disease populations suffer from small sample size, selection bias and heterogeneity. The databases provided herein from healthy populations solve these problems by permitting large sample bands, simple selection methods and diluted heterogeneity.

Provided herein are first databases of parameters, associated with non-selected, particularly healthy, subjects. Also provided are combinations of the databases with indexed samples obtained from each of the subjects. Further provided are databases produced from the first  
5 databases. These contain in addition to the original parameters information, such as genotypic information, including, but are not limited to, genomic sequence information, derived from the samples.

The databases, which are herein designated healthy databases, are so-designated because they are not obtained from subjects pre-selected  
10 for a particular disease. Hence, although individual members may have a disease, the collection of individuals is not selected to have a particular disease.

The subjects from whom the parameters are obtained comprise either a set of subjects who are randomly selected across, preferably, all  
15 populations, or are pre-selected to be disease-free or healthy. As a result, the database is not selected to be representative of any pre-selected phenotype, genotype, disease or other characteristic. Typically the number of subjects from which the database is prepared is selected to produce statistically significant results when used in the methods  
20 provided herein. Preferably, the number of subjects will be greater than 100, more preferably greater than 200, yet more preferably greater than 1000. The precise number can be empirically determined based upon the frequency of the parameter(s) that be used to sort the database. Generally the population can have at least 50, at least 100, at least 200,  
25 at least 500, at least 1000, at least 5000 or at least 10,000 or more subjects.

Upon identification of a collection of subjects, information about each subject is recorded and associated with each subject as a database. The information associated with each of the subjects, includes, but is not  
30 limited to, information related to historical characteristics of the subjects,

phenotypic characteristics and also genotypic characteristics, medical characteristics and any other traits and characteristics about the subject that can be determined. This information will serve as the basis for sorting the database.

- 5           In an exemplary embodiment, the subjects are mammals, such as humans, and the information relates to one or more of parameters, such as age, sex, medical history, ethnicity and any other factor. Such information, when the animals are humans, for example, can be obtained by a questionnaire, and by observations about the individual, such as hair
- 10 color, eye color and other characteristics. Genotypic information will be obtained from tissue or other body and body fluid samples from the subject.

- The healthy genomic database can include profiles and polymorphisms from healthy individuals from a library of blood samples
- 15 where each sample in the library is an individual and separate blood or other tissue sample. Each sample in the database is profiled as to the sex, age, ethnic group, and disease history of the donor.

- The databases are generated by first identifying healthy populations of subjects and obtaining information about each subject that will serve
- 20 as the sorting parameters for the database. This information is preferably entered into a storage medium, such as the memory of a computer.

- The information obtained about each subject in a population used for generating the database is stored in a computer memory or other suitable storage medium. The information is linked to an identifier
- 25 associated with each subject. Hence the database will identify a subject, for example by a datapoint representative of a bar code, and then all information, such as the information from a questionnaire, regarding the individual is associated with the datapoint. As the information is collected the database is generated.

Thus, for example, profile information, such as subject histories obtained from questionnaires, is collected in the database. The resulting database can be sorted as desired, using standard software, such as by age, sex and/or ethnicity. An exemplary questionnaire for subjects from  
5 whom samples are to be obtained is shown in Figures 22A-D. Each questionnaire preferably is identified by a bar code, particularly a machine readable bar code for entry into the database. After a subject provides data and is deemed to be healthy (*i.e.*, meets standards for blood donation), the data in the questionnaire is entered into the database and  
10 is associated with the bar code. A tissue, cell or blood sample is obtained from the subject.

Figure 4 exemplifies processing and tracking of blood sample components. Each component is tracked with a bar code, dated, is entered into the database and associated with the subject and the profile  
15 of the subject. Typically, the whole blood is centrifuged to produce plasma, red blood cells (which pellet) and leukocytes found in the buffy coat which layers in between. Various samples are obtained and coded with a bar code and stored for use as needed.

Samples are collected from the subjects. The samples include, but  
20 are not limited to, tissues, cells, and fluids, such as nucleic acid, blood, plasma, amniotic fluid, synovial fluid, urine, saliva, aqueous humor, sweat, sperm samples and cerebral spinal fluid. It is understood that the particular set of samples depends upon the organisms in the population.

Once samples are obtained the collection can be stored and, in  
25 preferred embodiments, each sample is indexed with an identifier, particularly a machine readable code, such as a bar code. For analyses, the samples or components of the samples, particularly biopolymers and small molecules, such as nucleic acids and/or proteins and metabolites, are isolated.

After samples are analyzed, this information is entered into the database in the memory of the storage medium and associated with each subject. This information includes, but is not limited to, genotypic information. Particularly, nucleic acid sequence information and other

5 information indicative of polymorphisms, such as masses of PCR fragments, peptide fragment sequences or masses, spectra of biopolymers and small molecules and other indicia of the structure or function of a gene, gene product or other marker from which the existence of a polymorphism within the population can be inferred.

10 In an exemplary embodiment, a database can be derived from a collection of blood samples. For example, Figure 1 (see, also Figure 10) shows the status of a collection of over 5000 individual samples. The samples were processed in the laboratory following SOP (standard operating procedure) guidelines. Any standard blood processing protocol  
15 may be used.

For the exemplary database described herein, the following criteria were used to select subjects:

No testing is done for infectious agents.

Age: At least 17 years old

20 Weight: Minimum of 110 pounds

Permanently Disqualified:

History of hepatitis (after age 11)

Leukemia Lymphoma

Human immunodeficiency virus (HIV), AIDS

25 Chronic kidney disease

Temporarily Disqualified:

Pregnancy - until six weeks after delivery, miscarriage or abortion

Major surgery or transfusions - for one year

Mononucleosis - until complete recovery

30 Prior whole blood donation - for eight weeks

Antibiotics by injection for one week; by mouth, for forty-eight hours, except antibiotics for skin complexion;

5 year Deferment:

Internal cancer and skin cancer if it has been removed, is healed

5 and there is no recurrence

These correspond to blood bank criteria for donating blood and represent a healthy population as defined herein for a human healthy database.

#### **Structure of the database**

Any suitable database structure and format known to those of skill  
 10 in the art may be employed. For example, a relational database is a preferred format in which data is stored as matrices or tables of the parameters linked by an indexer that identifies each subject. Software for preparing and manipulating, including sorting the database, can be readily developed or adapted from commercially available software, such as  
 15 Microsoft Access.

#### **Quality control**

Quality control procedures can be implemented. For example, after collection of samples, the quality of the collection in the bank can be assessed. For example, mix-up of samples can be checked by testing for  
 20 known markers, such as sex. After samples are separated by ethnicity, samples are randomly tested for a marker associated with a particular ethnicity, such as HLA DQA1 group specific component, to assess whether the samples have been properly sorted by ethnic group. An exemplary sample bank is depicted in Figure 4.

#### **25 Obtaining genotypic data and other parameters for the database**

After informational and historical parameters are entered into the database, material from samples obtained from each subject, is analyzed. Analyzed material include proteins, metabolites, nucleic acids, lipids and any other desired constituent of the material. For example, nucleic acids,  
 30 such as genomic DNA, can be analyzed by sequencing.

Sequencing can be performed using any method known to those of skill in the art. For example, if a polymorphism is identified or known, and it is desired to assess its frequency or presence among the subjects in the database, the region of interest from each sample can be isolated, such as by PCR or restriction fragments, hybridization or other suitable method known to those of skill in the art and sequenced. For purposes herein, sequencing analysis is preferably effected using mass spectrometry (see, *e.g.*, U.S. Patent Nos. 5,547,835, 5,622,824, 5,851,765, and 5,928,906). Nucleic acids can also be sequence by hybridization (see, *e.g.*, U.S. Patent Nos. 5,503,980, 5,631,134, 5,795,714) and including analysis by mass spectrometry (see, U.S. application Serial Nos. 08/419,994 and 09/395,409).

In other detection methods, it is necessary to first amplify prior to identifying the allelic variant. Amplification can be performed, *e.g.*, by PCR and/or LCR, according to methods known in the art. In one embodiment, genomic DNA of a cell is exposed to two PCR primers and amplification for a number of cycles sufficient to produce the required amount of amplified DNA. In preferred embodiments, the primers are located between 150 and 350 base pairs apart.

Alternative amplification methods include: self sustained sequence replication (Guatelli, J. C. et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:1874-1878), transcriptional amplification system (Kwoh, D. Y. et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:1173-1177), Q-Beta Replicase (Lizardi, P. M. et al., 1988, Bio/Technology 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

Nucleic acids can also be analyzed by detection methods and protocols, particularly those that rely on mass spectrometry (see, *e.g.*,

U.S. Patent No. 5,605,798, 6,043,031, allowed copending U.S. application Serial No. 08/744,481, U.S. application Serial No. 08/990,851 and International PCT application No. WO 99/31273, International PCT application No. WO 98/20019). These methods can be

5 automated (see, *e.g.*, copending U.S. application Serial No. 09/285,481 and published International PCT application No. PCT/US00/08111, which describes an automated process line). Preferred among the methods of analysis herein are those involving the primer oligo base extension (PROBE) reaction with mass spectrometry for detection (described herein

10 and elsewhere, see *e.g.*, U.S. Patent No. 6,043,031; see, also U.S. application Serial Nos. 09/287,681, 09/287,682, 09/287,141 and 09/287,679, allowed copending U.S. application Serial No. 08/744,481, International PCT application No. PCT/US97/20444, published as International PCT application No. WO 98/20019, and based upon U.S.

15 application Serial Nos. 08/744,481, 08/744,590, 08/746,036, 08/746,055, 08/786,988, 08/787,639, 08/933,792, 08/746,055, 08/786,988 and 08/787,639; see, also U.S. application Serial No. 09/074,936, U.S. Patent No. 6,024,925, and U.S. application Serial Nos. 08/746,055 and 08/786,988, and published International PCT application

20 No. WO 98/20020)

A preferred format for performing the analyses is a chip based format in which the biopolymer is linked to a solid support, such as a silicon or silicon-coated substrate, preferably in the form of an array. More preferably, when analyses are performed using mass spectrometry,

25 particularly MALDI, small nanoliter volumes of sample are loaded on, such that the resulting spot is about, or smaller than, the size of the laser spot. It has been found that when this is achieved, the results from the mass spectrometric analysis are quantitative. The area under the signals in the resulting mass spectra are proportional to concentration (when normalized

30 and corrected for background). Methods for preparing and using such

chips are described in U.S. Patent No. 6,024,925, co-pending U.S. application Serial Nos. 08/786,988, 09/364,774, 09/371,150 and 09/297,575; see, also U.S. application Serial No. PCT/US97/20195, which published as WO 98/20020. Chips and kits for performing these

- 5 analyses are commercially available from SEQUENOM under the trademark MassARRAY. MassArray relies on the fidelity of the enzymatic primer extension reactions combined with the miniaturized array and MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry to deliver results rapidly. It accurately distinguishes
- 10 single base changes in the size of DNA fragments associated with genetic variants without tags.

- The methods provided herein permit quantitative determination of alleles. The areas under the signals in the mass spectra can be used for quantitative determinations. The frequency is determined from the ratio
- 15 of the signal to the total area of all of the spectrum and corrected for background. This is possible because of the PROBE technology as described in the above applications incorporated by reference herein.

- Additional methods of analyzing nucleic acids include amplification-based methods including polymerase chain reaction (PCR), ligase chain
- 20 reaction (LCR), mini-PCR, rolling circle amplification, autocatalytic methods, such as those using Q $\beta$  replicase, TAS, 3SR, and any other suitable method known to those of skill in the art.

- Other methods for analysis and identification and detection of polymorphisms, include but are not limited to, allele specific probes,
- 25 Southern analyses, and other such analyses.

The methods described below provide ways to fragment given amplified or non-amplified nucleotide sequences thereby producing a set of mass signals when mass spectrometry is used to analyze the fragment mixtures.

Amplified fragments are yielded by standard polymerase chain methods (US 4,683,195 and 4,683,202). The fragmentation method involves the use of enzymes that cleave single or double strands of DNA and enzymes that ligate DNA. The cleavage enzymes can be glycosylases, nickases, and site-specific and non site-specific nucleases with the most preferred enzymes being glycosylases, nickases, and site-specific nucleases.

#### **Glycosylase Fragmentation Method**

DNA glycosylases specifically remove a certain type of nucleobase from a given DNA fragment. These enzymes can thereby produce abasic sites, which can be recognized either by another cleavage enzyme, cleaving the exposed phosphate backbone specifically at the abasic site and producing a set of nucleobase specific fragments indicative of the sequence, or by chemical means, such as alkaline solutions and or heat. The use of one combination of a DNA glycosylase and its targeted nucleotide would be sufficient to generate a base specific signature pattern of any given target region.

Numerous DNA glycosylases are known, For example, a DNA glycosylase can be uracil-DNA glycosylase (UDG) , 3-methyladenine DNA glycosylase, 3-methyladenine DNA glycosylase II, pyrimidine hydrate-DNA glycosylase, FaPy-DNA glycosylase, thymine mismatch-DNA glycosylase, hypoxanthine-DNA glycosylase, 5-Hydroxymethyluracil DNA glycosylase (HmUDG), 5-Hydroxymethylcytosine DNA glycosylase, or 1,N6-etheno-adenine DNA glycosylase (see, *e.g.*, U.S. Patent Nos. 5,536,649, 5,888,795, 5,952,176 and 6,099,553, International PCT application Nos. WO 97/03210, WO 99/54501; see, also, Eftedal et al. (1993) Nucleic Acids Res 21:2095-2101, Bjelland and Seeberg (1987) Nucleic Acids Res. 15:2787-2801, Saparbaev et al. (1995) Nucleic Acids Res. 23:3750-3755, Bessho (1999) Nucleic Acids Res. 27:979-983) corresponding to the enzyme's modified nucleotide or nucleotide analog target. A preferred glycosylase is uracil-DNA glycosylase (UDG).

Uracil, for example, can be incorporated into an amplified DNA molecule by amplifying the DNA in the presence of normal DNA precursor nucleotides (e.g. dCTP, dATP, and dGTP) and dUTP. When the amplified product is treated with UDG, uracil residues are cleaved. Subsequent

5 chemical treatment of the products from the UDG reaction results in the cleavage of the phosphate backbone and the generation of nucleobase specific fragments. Moreover, the separation of the complementary strands of the amplified product prior to glycosylase treatment allows complementary patterns of fragmentation to be generated. Thus, the use

10 of dUTP and Uracil DNA glycosylase allows the generation of T specific fragments for the complementary strands, thus providing information on the T as well as the A positions within a given sequence. Similar to this, a C-specific reaction on both (complementary) strands (i.e. with a C-specific glycosylase) yields information on C as well as G positions within

15 a given sequence if the fragmentation patterns of both amplification strands are analyzed separately. Thus, with the glycosylase method and mass spectrometry, a full series of A, C, G and T specific fragmentation patterns can be analyzed.

#### **Nickase Fragmentation Method**

20 A DNA nickase, or DNase, can be used recognize and cleave one strand of a DNA duplex. Numerous nickases are known. Among these, for example, are nickase NY2A nickase and NYS1 nickase (Megabase) with the following cleavage sites:

NY2A: 5'...R AG...3'

25 3'...Y TC...5' where R = A or G and Y = C or T

NYS1: 5'... CC[A/G/T]...3'

3'... GG[T/C/A]...5'.

#### **Fen-Ligase Fragmentation Method**

The Fen-ligase method involves two enzymes: Fen-1 enzyme and a

30 ligase.

The Fen-1 enzyme is a site-specific nuclease known as a "flap" endonuclease (US 5,843,669, 5,874,283, and 6,090,606). This enzymes recognizes and cleaves DNA "flaps" created by the overlap of two oligonucleotides hybridized to a target DNA strand. This cleavage is

5 highly specific and can recognize single base pair mutations, permitting detection of a single homologue from an individual heterozygous at one SNP of interest and then genotyping that homologue at other SNPs occurring within the fragment. Fen-1 enzymes can be Fen-1 like nucleases e.g. human, murine, and *Xenopus* XPG enzymes and yeast RAD2

10 nucleases or Fen-1 endonucleases from, for example, *M. jannaschii*, *P. furiosus*, and *P. woesei*. Among preferred enzymes are the Fen-1 enzymes.

The ligase enzyme forms a phosphodiester bond between two double stranded nucleic acid fragments. The ligase can be DNA Ligase I or DNA Ligase III (see, *e.g.*, U.S. Patent Nos. US 5,506,137, 5,700,672, 5,858,705 and 5,976,806; see, also, Waga, *et al.* (1994) J. Biol. Chem. 269:10923-10934, Li et al. (1994) Nucleic Acids Res. 22:632-638, Arrand et al. (1986) J. Biol. Chem. 261:9079-9082, Lehman (1974) Science 186:790-797, Higgins and Cozzarelli (1979) Methods Enzymol.

15 68:50-71, Lasko et al. (1990) Mutation Res. 236:277-287, and Lindahl and Barnes (1992) Ann. Rev. Biochem. 61:251-281). Thermostable ligase (Epicenter Technologies), where "thermostable" denotes that the ligase retains activity even after exposure to temperatures necessary to separate two strands of DNA, are among preferred ligases for use herein.

20

## 25 **Type IIS Enzyme Fragmentation Method**

Restriction enzymes bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular recognition sequence. These enzymes have been classified into three groups (e.g. Types I, II, and III) as known to those of skill in the art. Because of the

30 properties of type I and type III enzymes, they have not been widely used

in molecular biological applications. Thus, for this invention type II enzymes are preferred. Of the thousands of restriction enzymes known in the arts, there are 179 different type II specificities. Of the 179 unique type II restriction endonucleases, 31 have a 4-base recognition sequence, 11 have a 5-base recognition sequence, 127 have a 6-base recognition sequence, and 10 have recognition sequences of greater than six bases (US 5,604,098). Of category type II enzymes, type IIS is preferred.

Type IIS enzymes can be *Alw* XI, *Bbv* I, *Bce* 83, *Bpm* I, *Bsg* I, *Bsm* AI, *Bsm* FI, *Bsa* I, *Bcc* I, *Bcg* I, *Ear* I, *Eco* 57I, *Esp* 3I, *Fau* I, *Fok* I, *Gsu* I, *Hga* I, *Mme* I, *Mbo* II, *Sap* I, and the like. The preferred type IIS enzyme is *Fok* I.

The *Fok* I enzyme endonuclease is an exemplary well characterized member of the Type IIS class (see, e.g., U.S. Patent Nos. 5,714,330, 5,604,098, 5,436,150, 6,054,276 and 5,871,911; see, also, Szybalski et al. (1991) Gene 100:13-26, Wilson and Murray (1991) Ann. Rev. Genet. 25:585-627, Sugisaki et al. (1981) Gene 16:73-78, Podhajaska and Szalski (1985) Gene 40:175-182. *Fok* I recognizes the sequence 5'GGATG-3' and cleaves DNA accordingly. Type IIS restriction sites can be introduced into DNA targets by incorporating the site into primers used to amplify such targets. Fragments produced by digestion with *Fok* I are site specific and can be analyzed by mass spectrometry methods such as MALDI-TOF mass spectrometry, ESI-TOF mass spectrometry, and any other type of mass spectrometry well known to those of skill in the art.

Once a polymorphism has been found to correlate with a parameter such as age. The possibility of false results due to allelic dropout is examined by doing comparative PCR in an adjacent region of the genome.

### Analyses

In using the database, allelic frequencies can be determined across the population by analyzing each sample in the population individually, determining the presence or absence of allele or marker of interest in each individual sample, and then determining the frequency of the marker in the population. The database can then be sorted (stratified) to identify any correlations between the allele and a selected parameter using standard statistical analysis. If a correlation is observed, such as a decrease in a particular marker with age or correlation with sex or other parameter, then the marker is a candidate for further study, such as genetic mapping to identify a gene or pathway in which it is involved. The marker may then be correlated, for example, with a disease. Haplotyping can also be carried out. Genetic mapping can be effected using standard methods and may also require use of databases of others, such as databases previously determined to be associated with a disorder.

Exemplary analyses have been performed and these are shown in the figures, and discussed herein.

### Sample pooling

It has been found that using the databases provided herein, or any other database of such information, substantially the same frequencies that were obtained by examining each sample separately can be obtained by pooling samples, such as in batches of 10, 20, 50, 100, 200, 500, 1000 or any other number. A precise number may be determined empirically if necessary, and can be as low as 3.

In one embodiment, the frequency of genotypic and other markers can be obtained by pooling samples. To do this a target population and a genetic variation to be assessed is selected, a plurality of samples of biopolymers are obtained from members of the population, and the biopolymer from which the marker or genotype can be inferred is

determined or detected. A comparison of samples tested in pools and individually and the sorted results therefrom are shown in Figure 9, which shows frequency of the factor VII Allele 353Q. Figure 10 depicts the frequency of the CETP Allele CETP in pooled versus individual samples.

- 5 Figure 15 shows ethnic diversity among various ethnic groups in the database using pooled DNA samples to obtain the data. Figures 12-14 show mass spectra for these samples.

Pooling of test samples has application not only to the healthy databases provided herein, but also to use in gathering data for entry into  
10 any database of subjects and genotypic information, including typical databases derived from diseased populations. What is demonstrated herein, is the finding that the results achieved are statistically the same as the results that would be achieved if each sample is analyzed separately. Analysis of pooled samples by a method, such as the mass spectrometric  
15 methods provided herein, permits resolution of such data and quantitation of the results.

For factor VII the R53Q acid polymorphism was assessed. In Figure 9, the "individual" data represent allelic frequency observed in 92 individuals reactions. The pooled data represent the allelic frequency of  
20 the same 92 individuals pooled into a single probe reaction. The concentration of DNA in the samples of individual donors is 250 nanograms. The total concentration of DNA in the pooled samples is also 250 nanograms, where the concentration of any individual DNA is 2.7 nanograms.

- 25 It also was shown that it is possible to reduce the DNA concentration of individuals in a pooled samples from 2.7 nanograms to 0.27 nanograms without any change in the quality of the spectrum or the ability to quantitate the amount of sample detected. Hence low concentrations of sample may be used in the pooling methods.

### Use of the databases and markers identified thereby

The successful use of genomics requires a scientific hypothesis (*i.e.*, common genetic variation, such as a SNP), a study design (*i.e.*, complex disorders), samples and technology, such as the chip-based  
5 mass spectrometric analyses (see, *e.g.*, U.S. Patent No. 5,605,798, U.S. Patent No. 5,777,324, U.S. Patent No. 6,043,031, allowed copending U.S. application Serial No. 08/744,481, U.S. application Serial No. 08/990,851, International PCT application No. WO 98/20019, copending U.S. application Serial No. 09/285,481, which describes an automated  
10 process line for analyses; see, also, U.S. application Serial Nos. 08/617,256, 09/287,681, 09/287,682, 09/287,141 and 09/287,679, allowed copending U.S. application Serial No. 08/744,481, International PCT application No. PCT/US97/20444, published as International PCT application No. WO 98/20019, and based upon U.S. application Serial  
15 Nos. 08/744,481, 08/744,590, 08/746,036, 08/746,055, 08/786,988, 08/787,639, 08/933,792, 08/746,055, 09/266,409, 08/786,988 and 08/787,639; see, also U.S. application Serial No. 09/074,936). All of these aspects can be used in conjunction with the databases provided herein and samples in the collection.

20 The databases and markers identified thereby can be used, for example, for identification of previously unidentified or unknown genetic markers and to identify new uses for known markers. As markers are identified, these may be entered into the database to use as sorting parameters from which additional correlations may be determined.

### 25 Previously unidentified or unknown genetic markers

The samples in the healthy databases can be used to identify new polymorphisms and genetic markers, using any mapping, sequencing, amplification and other methodologies, and in looking for polymorphisms among the population in the database. The thus-identified polymorphism  
30 can then be entered into the database for each sample, and the database

sorted (stratified) using that polymorphism as a sorting parameter to identify any patterns and correlations that emerge, such as age correlated changes in the frequency of the identified marker. If a correlation is identified, the locus of the marker can be mapped and its function or

5 effect assessed or deduced.

Thus, the databases here provide means for:

identification of significantly different allelic frequencies of genetic factors by comparing the occurrence or disappearance of the markers with increasing age in population and then associating the markers with a  
10 disease or a biochemical pathway;

identification of significantly different allelic frequencies of disease causing genetic factors by comparing the male with the female population or comparing other selected stratified populations and associating the markers with a disease or a biochemical pathway;

15 identification of significantly different allelic frequencies of disease causing genetic factors by comparing different ethnic groups and associating the markers with a disease or a biochemical pathway that is known to occur in high frequency in the ethnic group;

profiling potentially functional variants of genes through the general  
20 panmixed population stratified according to age, sex, and ethnic origin and thereby demonstrating the contribution of the variant genes to the physical condition of the investigated population;

identification of functionally relevant gene variants by gene disequilibrium analysis performed within the general panmixed population  
25 stratified according to age, sex, and ethnic origin and thereby demonstrating their contribution to the physical condition of investigated population;

identification of potentially functional variants of chromosomes or parts of chromosomes by linkage disequilibrium analysis performed within  
30 the general panmixed population stratified according to age, sex, and

ethnic origin and thereby demonstrating their contribution to the physical condition of investigated population.

#### **Uses of the identified markers and known markers**

- The databases may also be used in conjunction with known
- 5 markers and sorted to identify any correlations. For example, the databases can be used for:
- determination and evaluation of the penetrance of medically relevant polymorphic markers;
  - determination and evaluation of the diagnostic specificity of
  - 10 medically relevant genetic factors;
  - determination and evaluation of the positive predictive value of medically relevant genetic factors;
  - determination and evaluation of the onset of complex diseases, such as, but are not limited to, diabetes, hypertension, autoimmune
  - 15 diseases, arteriosclerosis, cancer and other diseases within the general population with respect to their causative genetic factors;
  - delineation of the appropriate strategies for preventive disease treatment;
  - delineation of appropriate timelines for primary disease intervention;
  - 20 validation of medically relevant genetic factors identified in isolated populations regarding their general applicability;
  - validation of disease pathways including all potential target structures identified in isolated populations regarding their general applicability; and
  - 25 validation of appropriate drug targets identified in isolated populations regarding their general applicability.
- Among the diseases and disorders for which polymorphisms may be linked include, those linked to inborn errors of metabolism, acquired metabolic disorders, intermediary metabolism, oncogenesis pathways,
- 30 blood clotting pathways, and DNA synthetic and repair pathways DNA

repair/replication/transcription factors and activities, *e.g.*, such as genes related to oncogenesis, aging and genes involved in blood clotting and the related biochemical pathways that are related to thrombosis, embolism, stroke, myocardial infarction, angiogenesis and oncogenesis.

- 5       For example, a number of diseases are caused by or involve deficient or defective enzymes in intermediary metabolism (see, e.g., Tables 1 and 2, below) that result, upon ingestion of the enzyme substrates, in accumulation of harmful metabolites that damage organs and tissues, particularly an infant's developing brain and other organs,  
10       resulting in mental retardation and other developmental disorders.  
**Identification of markers and genes for such disorders is of great interest.**

#### **Model systems**

- Several gene systems, p21, p53 and Lipoprotein Lipase polymorphism (N291S), were selected. The p53 gene is a tumor  
15       suppressor gene that is mutated in diverse tumor types. One common allelic variant occurs at codon 72. A polymorphism that has been identified in the p53 gene, i.e., the R72P allele, results in an amino acid exchange, arginine to proline, at codon 72 of the gene.

- Using diseased populations, it has been shown that there are ethnic  
20       differences in the allelic distribution of these alleles among African-Americans and Caucasians in the U.S. The results here support this finding and also demonstrate that the results obtained with a healthy database are meaningful (see, Figure 7B).

- The 291S allele leads to reduced levels of high density lipoprotein  
25       cholesterol (HDL-C) that is associated with an increased risk of males for arteriosclerosis and in particular myocardial infarction (see, Reymer *et al.* (1995) *Nature Genetics* 10:28-34).

- Both genetic polymorphisms were profiled within a part of the Caucasian population-based sample bank. For the polymorphism located  
30       in the lipoprotein lipase gene a total of 1025 unselected individuals (436

males and 589 females) were tested. Genomic DNA was isolated from blood samples obtained from the individuals.

As shown in the Examples and figures, an exemplary database containing about 5000 subjects, answers to the questionnaire (see Figure 3), and genotypic information has been stratified. A particular known allele has been selected, and the samples tested for the marker using mass spectrometric analyses, particularly PROBE (see the EXAMPLES) to identify polymorphisms in each sample. The population in the database has been sorted according to various parameters and correlations have been observed. For example, FIGURES 2A-C, show sorting of the data by age and sex for the Lipoprotein Lipase gene in the Caucasian population in the database. The results show a decrease in the frequency of the allele with age in males but no such decrease in females. Other alleles that have been tested against the database, include, alleles of p53, p21 and factor VII. Results when sorted by age are shown in the figures.

These examples demonstrate an effect of altered frequency of disease causing genetic factors within the general population. The scientific interpretation of those results allows prediction of medical relevance of polymorphic genetic alterations. In addition, conclusions can be drawn with regard to their penetrance, diagnostic specificity, positive predictive value, onset of disease, most appropriate onset of preventive strategies, and the general applicability of genetic alterations identified in isolated populations to panmixed populations.

Therefore, an age- and sex-stratified population-based sample bank that is ethnically homogenous is a suitable tool for rapid identification and validation of genetic factors regarding their potential medical utility.

**Exemplary computer system for creating, storing and processing the databases**

## Systems

Systems, including computers, containing the databases are provided herein. The computers and databases can be used in

- 5 conjunction, for example, with the APL system (see, copending U.S. application Serial No. 09/285,481), which is an automated system for analyzing biopolymers, particularly nucleic acids. Results from the APL system can be entered into the database.

- Any suitable computer system may be used. The computer system  
10 may be integrated into systems for sample analysis, such as the automated process line described herein (see, *e.g.*, copending U.S. application Serial No. 09/285,481).

- Figure 17 is a block diagram of a computer constructed in to provide and process the databases described herein. The processing that  
15 maintains the database and performs the methods and procedures may be performed on multiple computers all having a similar construction, or may be performed by a single, integrated computer. For example, the computer through which data is added to the database may be separate from the computer through which the database is sorted, or may be  
20 integrated with it. In either arrangement, the computers performing the processing may have a construction as illustrated in Figure 17.

- Figure 17 is a block diagram of an exemplary computer 1700 that maintains the database described above and performs the methods and procedures. Each computer 1700 operates under control of a central  
25 processor unit (CPU) 1702, such as a "Pentium" microprocessor and associated integrated circuit chips, available from Intel Corporation of Santa Clara, California, USA. A computer user can input commands and data from a keyboard and display mouse 1704 and can view inputs and computer output at a display 1706. The display is typically a video  
30 monitor or flat panel display device. The computer 1700 also includes a direct access storage device (DASD) 1707, such as a fixed hard disk

drive. The memory 1708 typically comprises volatile semiconductor random access memory (RAM). Each computer preferably includes a program product reader 1710 that accepts a program product storage device 1712, from which the program product reader can read data (and to which it can optionally write data). The program product reader can comprise, for example, a disk drive, and the program product storage device can comprise removable storage media such as a magnetic floppy disk, an optical CD-ROM disc, a CD-R disc, a CD-RW disc, or a DVD data disc. If desired, the computers can be connected so they can communicate with each other, and with other connected computers, over a network 1713. Each computer 1700 can communicate with the other connected computers over the network 1713 through a network interface 1714 that enables communication over a connection 1716 between the network and the computer.

The computer 1700 operates under control of programming steps that are temporarily stored in the memory 1708 in accordance with conventional computer construction. When the programming steps are executed by the CPU 1702, the pertinent system components perform their respective functions. Thus, the programming steps implement the functionality of the system as described above. The programming steps can be received from the DASD 1707, through the program product reader 1712, or through the network connection 1716. The storage drive 1710 can receive a program product, read programming steps recorded thereon and transfer the programming steps into the memory 1708 for execution by the CPU 1702. As noted above, the program product storage device 1710 can comprise any one of multiple removable media having recorded computer-readable instructions, including magnetic floppy disks and CD-ROM storage discs. Other suitable program product storage devices can include magnetic tape and semiconductor memory

chips. In this way, the processing steps necessary for operation can be embodied on a program product.

Alternatively, the program steps can be received into the operating memory 1708 over the network 1713. In the network method, the  
5 computer receives data including program steps into the memory 1708 through the network interface 1714 after network communication has been established over the network connection 1716 by well-known methods that will be understood by those skilled in the art without further explanation. The program steps are then executed by the CPU 1702 to  
10 implement the processing of the Garment Database system.

It should be understood that all of the computers of the system preferably have a construction similar to that shown in Figure 17, so that details described with respect to the Figure 17 computer 1700 will be understood to apply to all computers of the system 1700. This is  
15 indicated by multiple computers 1700 shown connected to the network 1713. Any one of the computers 1700 can have an alternative construction, so long as they can communicate with the other computers and support the functionality described herein.

Figure 18 is a flow diagram that illustrates the processing steps  
20 performed using the computer illustrated in Figure 17, to maintain and provide access to the databases, such as for identifying polymorphic genetic markers. In particular, the information contained in the database is stored in computers having a construction similar to that illustrated in Figure 17. The first step for maintaining the database, as indicated in  
25 Figure 18, is to identify healthy members of a population. As noted above, the population members are subjects that are selected only on the basis of being healthy, and where the subjects are mammals, such as humans, they are preferably selected based upon apparent health and the absence of detectable infections. The step of identifying is represented  
30 by the flow diagram box numbered 1802.

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The next step, represented by the flow diagram box numbered 1804, is to obtain identifying and historical information and data relating to the identified members of the population. The information and data comprise parameters for each of the population members, such as

5 member age, ethnicity, sex, medical history, and ultimately genotypic information. Initially, the parameter information is obtained from a questionnaire answered by each member, from whom a body tissue or body fluid sample also is obtained. The step of entering and storing these parameters into the database of the computer is represented by the flow

10 diagram box numbered 1806. As additional information about each population member and corresponding sample is obtained, this information can be inputted into the database and can serve as a sorting parameter.

In the next step, represented by the flow diagram box numbered

15 1808, the parameters of the members are associated with an indexer. This step may be executed as part of the database storage operation, such as when a new data record is stored according to the relational database structure and is automatically linked with other records according to that structure. The step 1806 also may be executed as part

20 of a conventional data sorting or retrieval process, in which the database entries are searched according to an input search or indexing key value to determine attributes of the data. For example, such search and sort techniques may be used to follow the occurrence of known genetic markers and then determine if there is a correlation with diseases for

25 which they have been implicated. Examples of this use are for assessing the frequencies of the p53 and Lipoprotein Lipase polymorphisms.

Such searching of the database also may be valuable for identifying one or more genetic markers whose frequency changes within the population as a function of age, ethnic group, sex, or some other criteria.

30 This can allow the identification of previously unknown polymorphisms

and, ultimately, identification of a gene or pathway involved in the onset and progression of disease.

In addition, the database can be used for taking an identified polymorphism and ascertaining whether it changes in frequency when the data is sorted according to a selected parameter.

In this way, the databases and methods provided herein permit, among other things, identification of components, particularly key components, of a disease process by understanding its genetic underpinnings, and also an understanding of processes, such as individual drug responses. The databases and methods provided herein also can be used in methods involving elucidation of pathological pathways, in developing new diagnostic assays, identifying new potential drug targets, and in identifying new drug candidates.

**Morbidity and/or early mortality associated polymorphisms**

A database containing information provided by a population of healthy blood donors who were not selected for any particular disease to can be used to identify polymorphisms and the alleles in which they are present, whose frequency decreases with age. These may represent morbidity susceptibility markers and genes.

Polymorphisms of the genome can lead to altered gene function, protein function or genome instability. To identify those polymorphisms which have a clinical relevance/utility is the goal of a world-wide scientific effort. It can be expected that the discovery of such polymorphisms will have a fundamental impact on the identification and development of novel drug compounds to cure diseases. However, the strategy to identify valuable polymorphisms is cumbersome and dependent upon the availability of many large patient and control cohorts to show disease association. In particular, genes that cause a general risk of the population to suffer from any disease (morbidity susceptibility genes) will escape these case/control studies entirely.

- Here described is a screening strategy to identify morbidity susceptibility genes underlying a variety of different diseases. The definition of a morbidity susceptibility gene is a gene that is expressed in many different cell types or tissues (housekeeping gene) and its altered
- 5 function can facilitate the expression of a clinical phenotype caused by disease-specific susceptibility genes that are involved in a pathway specific for this disorder. In other words, these morbidity susceptibility genes predispose people to develop a distinct disease according to their genetic make-up for this disease.
- 10 Candidates for morbidity susceptibility genes can be found at the bottom level of pathways involving transcription, translation, heat-shock proteins, protein trafficking, DNA repair, assembly systems for subcellular structures (e.g. mitochondria, peroxysomes and other cellular
- 15 pathways control the quality of life at the cellular level as well as for the entire organism. Mutations/polymorphisms located in genes encoding proteins for those pathways can reduce the fitness of cells and make the organism more susceptible to express the clinical phenotype caused by the action of a disease-specific susceptibility gene. Therefore, these
- 20 morbidity susceptibility genes can be potentially involved in a whole variety of different complex diseases if not in all. Disease-specific susceptibility genes are involved in pathways that can be considered as disease-specific pathways like glucose-, lipid, hormone metabolism, etc.
- 25 The exemplified method permit, among other things, identification of genes and/or gene products involved in a man's general susceptibility to morbidity and/or mortality; use of these genes and/or gene products in studies to elucidate the genetic underpinnings of human diseases; use of these genes and/or gene products in combinatorial statistical analyses without or together with disease-specific susceptibility genes; use of
- 30 these genes and/or gene products to predict penetrance of disease

susceptibility genes; use of these genes and/or gene products in predisposition and/or acute medical diagnostics and use of these genes and/or gene products to develop drugs to cure diseases and/or to extend the life span of humans.

## 5 SCREENING PROCESS

The healthy population stratified by age, gender and ethnicity, etc. is a very efficient and a universal screening tool for morbidity associated genes. Changes of allelic frequencies in the young compared to the old population are expected to indicate putative morbidity susceptibility genes. Individual samples of this healthy population base can be pooled to further increase the throughput. In a proof of principle experiment pools of young and old Caucasian females and males were applied to screen more than 400 randomly chosen single nucleotide polymorphisms located in many different genes. Candidate polymorphisms were identified if the allelic difference was greater than 8% between young and old for both or only one of the genders. The initial results were assayed again in at least one independent subsequent experiments. Repeated experiments are necessary to recognize unstable biochemical reactions, which occur with a frequency of about 2-3% and can mimic age-related allelic frequency differences. Average frequency differences and standard deviations are calculated after successful reproducibility of initial results. The final allelic frequency is then compared to a reference population of Caucasian CEPH sample pool. The result should show similar allelic frequencies in the young Caucasian population. Subsequently, the exact allele frequencies of candidates including genotype information were obtained by analyzing all individual samples. This procedure is straight forward with regard to time and cost. It enables the screening of an enormous number of SNPs. So far, several markers with a highly significant association to age were identified and described below.

- In general at least 5 individual in a stratified population need to be screened to produce statistically significant results. The frequency of the allele is determined for an age stratified population. Chi square analysis is then performed on the allelic frequencies to determine if the difference
- 5 between age groups is statistically significant. A p value less than of 0.1 is considered to represent a statistically significant difference. More preferably the p value should be less than 0.05.

#### **Clinical Trials**

- The identification of markers whose frequency in a population
- 10 decreases with age also allows for better designed and balanced clinical trials. Currently, if a clinical trial utilizes a marker as a significant endpoint in a study and the marker disappears with age, then the results of the study may be inaccurate. By using methods provided herein, it can be ascertained that if a marker decreases in frequency with age. This
- 15 information considered and controlled when designing the study. For, example, an age independent marker could be substituted in its place.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

#### **EXAMPLE 1**

- 20 This example describes the use of a database containing information provided by a population of healthy blood donors who were not selected for any particular disease to determine the distribution of allelic frequencies of known genetic markers with age and by sex in a Caucasian subpopulation of the database. The results described in this
- 25 example demonstrate that a disease-related genetic marker or polymorphism can be identified by sorting a healthy database by a parameter or parameters, such as age, sex and ethnicity.

### Generating a database

Blood was obtained by venous puncture from human subjects who met blood bank criteria for donating blood. The blood samples were preserved with EDTA at pH 8.0 and labeled. Each donor provided  
5 information such as age, sex, ethnicity, medical history and family medical history. Each sample was labeled with a barcode representing identifying information. A database was generated by entering, for each donor, the subject identifier and information corresponding to that subject into the memory of a computer storage medium using commercially  
10 available software, e.g., Microsoft Access.

### Model genetic markers

The frequencies of polymorphisms known to be associated at some level with disease were determined in a subpopulation of the subjects represented in the database. These known polymorphisms occur in the  
15 p21, p53 and Lipoprotein Lipase genes. Specifically, the N291S polymorphism (N291S) of the Lipoprotein Lipase gene, which results in a substitution of a serine for an asparagine at amino acid codon 291, leads to reduced levels of high density lipoprotein cholesterol (HDL-C) that is associated with an increased risk of males for arteriosclerosis and in  
20 particular myocardial infarction (see, Reymer *et al.* (1995) *Nature Genetics* 10:28-34).

The p53 gene encodes a cell cycle control protein that assesses DNA damage and acts as a transcription factor regulating genes that control cell growth, DNA repair and apoptosis (programmed cell death).  
25 Mutations in the p53 gene have been found in a wide variety of different cancers, including different types of leukemia, with varying frequency. The loss of normal p53 function results in genomic instability and uncontrolled cell growth. A polymorphism that has been identified in the p53 gene, i.e., the R72P allele, results in the substitution of a proline for  
30 an arginine at amino acid codon 72 of the gene.

- The p21 gene encodes a cyclin-dependent kinase inhibitor associated with G1 phase arrest of normal cells. Expression of the p21 gene triggers apoptosis. Polymorphisms of the p21 gene have been associated with Wilms' tumor, a pediatric kidney cancer. One
- 5 polymorphism of the p21 gene, the S31R polymorphism, results in a substitution of an arginine for a serine at amino acid codon 31.

#### **Database analysis**

##### **Sorting of subjects according to specific parameters**

- The genetic polymorphisms were profiled within segments of the
- 10 Caucasian subpopulation of the sample bank. For p53 profiling, the genomic DNA isolated from blood from a total of 1277 Caucasian subjects age 18-59 years and 457 Caucasian subjects age 60-79 years was analyzed. For p21 profiling, the genomic DNA isolated from blood from a total of 910 Caucasian subjects age 18-49 years and 824
- 15 Caucasian subjects age 50-79 years was analyzed. For lipoprotein lipase gene profiling, the genomic DNA from a total of 1464 Caucasian females and 1470 Caucasian males under 60 years of age and a total of 478 Caucasian females and 560 Caucasian males over 60 years of age was analyzed.

##### **Isolation and analysis of genomic DNA**

- Genomic DNA was isolated from blood samples obtained from the individuals. Ten milliliters of whole blood from each individual was centrifuged at 2000 x g. One milliliter of the buffy coat was added to 9 ml of 155 mM  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$ , and 0.1 mM  $\text{Na}_2\text{EDTA}$ , incubated
- 25 10 min at room temperature and centrifuged for 10 min at 2000 x g. The supernatant was removed, and the white cell pellet was washed in 155 mM  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$  and 0.1 mM  $\text{Na}_2\text{EDTA}$  and resuspended in 4.5 ml of 50 mM Tris, 5 mM EDTA and 1% SDS. Proteins were precipitated from the cell lysate by 6 mM ammonium acetate, pH 7.3, and then
- 30 separated from the nucleic acids by centrifugation at 3000 x g. The

nucleic acid was recovered from the supernatant by the addition of an equal volume of 100% isopropanol and centrifugation at 2000 x g. The dried nucleic acid pellet was hydrated in 10 mM Tris, pH 7.6, and 1 mM Na<sub>2</sub>EDTA and stored at 4° C.

- 5 Assays of the genomic DNA to determine the presence or absence of the known genetic markers were developed using the BiomassPROBE™ detection method (primer oligo base extension) reaction. This method uses a single detection primer followed by an oligonucleotide extension step to give products, which can be readily resolved by mass
- 10 spectrometry, and, in particular, MALDI-TOF mass spectrometry. The products differ in length depending on the presence or absence of a polymorphism. In this method, a detection primer anneals adjacent to the site of a variable nucleotide or sequence of nucleotides and the primer is extended using a DNA polymerase in the presence of one or more
- 15 dideoxynTPs and, optionally, one or more deoxyNTPs. The resulting products are resolved by MALDI-TOF mass spectrometry. The mass of the products as measured by MALDI-TOF mass spectrometry makes possible the determination of the nucleotide(s) present at the variable site.

- First, each of the Caucasian genomic DNA samples was subjected
- 20 to nucleic acid amplification using primers corresponding to sites 5' and 3' of the polymorphic sites of the p21 (S31R allele), p53 (R72P allele) and Lipoprotein Lipase (N291S allele) genes. One primer in each primer pair was biotinylated to permit immobilization of the amplification product to a solid support. Specifically, the polymerase chain reaction primers used
  - 25 for amplification of the relevant segments of the p21, p53 and lipoprotein lipase genes are shown below: US4p21c31-2F (SEQ ID NO: 9) and US5p21-2R (SEQ ID NO: 10) for p21 gene amplification; US4-p53-ex4-F (also shown as p53-ex4US4 (SEQ ID NO: 2)) and US5-p53/2-4R (also shown as US5P53/4R (SEQ ID NO: 3)) for p53 gene amplification; and

US4-LPL-F2 (SEQ ID NO: 16) and US5-LPL-R2 (SEQ ID NO: 17) for lipoprotein lipase gene amplification.

Amplification of the respective DNA sequences was conducted according to standard protocols. For example, primers may be used in a concentration of 8 pmol. The reaction mixture (e.g., total volume 50  $\mu$ l) may contain Taq-polymerase including 10x buffer and dTNPs. Cycling conditions for polymerase chain reaction amplification may typically be initially 5 min. at 95°C, followed by 1 min. at 94°C, 45 sec at 53°C, and 30 sec at 72°C for 40 cycles with a final extension time of 5 min at 72°C. Amplification products may be purified by using Qiagen's PCR purification kit (No. 28106) according to manufacturer's instructions. The elution of the purified products from the column can be done in 50  $\mu$ l TE-buffer (10mM Tris, 1 mM EDTA, pH 7.5).

The purified amplification products were immobilized via a biotin-avidin linkage to streptavidin-coated beads and the double-stranded DNA was denatured. A detection primer was then annealed to the immobilized DNA using conditions such as, for example, the following: 50  $\mu$ l annealing buffer (20 mM Tris, 10 mM KCl, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 2 mM  $\text{MgSO}_4$ , 1% Triton X-100, pH 8) at 50°C for 10 min, followed by washing of the beads three times with 200  $\mu$ l washing buffer (40 mM Tris, 1 mM EDTA, 50 mM NaCl, 0.1% Tween 20, pH 8.8) and once in 200  $\mu$ l TE buffer.

The PROBE extension reaction was performed, for example, by using some components of the DNA sequencing kit from USB (No. 70770) and dNTPs or ddNTPs from Pharmacia. An exemplary protocol could include a total reaction volume of 45  $\mu$ l, containing of 21  $\mu$ l water, 6  $\mu$ l Sequenase-buffer, 3  $\mu$ l 10 mM DTT solution, 4.5  $\mu$ l, 0.5 mM of three dNTPs, 4.5  $\mu$ l, 2 mM the missing one ddNTP, 5.5  $\mu$ l glycerol enzyme dilution buffer, 0.25  $\mu$ l Sequenase 2.0, and 0.25 pyrophosphatase. The reaction can then by pipetted on ice and incubated for 15 min at room temperature and for 5 min at 37°C. The beads may be washed three

times with 200  $\mu$ l washing buffer and once with 60  $\mu$ l of a 70 mM  $\text{NH}_4$ -Citrate solution.

The DNA was denatured to release the extended primers from the immobilized template. Each of the resulting extension products was  
 5 separately analyzed by MALDI-TOF mass spectrometry using 3-hydroxypicolinic acid (3-HPA) as matrix and a UV laser.

Specifically, the primers used in the PROBE reactions are as shown below: P21/31-3 (SEQ ID NO: 12) for PROBE analysis of the p21 polymorphic site; P53/72 (SEQ ID NO: 4) for PROBE analysis of the p53  
 10 polymorphic site; and LPL-2 for PROBE analysis of the lipoprotein lipase gene polymorphic site. In the PROBE analysis of the p21 polymorphic site, the extension reaction was performed using dideoxy-C. The products resulting from the reaction conducted on a "wild-type" allele template (wherein codon 31 encodes a serine) and from the reaction  
 15 conducted on a polymorphic S31R allele template (wherein codon 31 encodes an arginine) are shown below and designated as P21/31-3 Ser (wt) (SEQ ID NO: 13) and P21/31-3 Arg (SEQ ID NO: 14), respectively. The masses for each product as can be measured by MALDI-TOF mass spectrometry are also provided (i.e., 4900.2 Da for the wild-type product  
 20 and 5213.4 Da for the polymorphic product).

In the PROBE analysis of the p53 polymorphic site, the extension reaction was performed using dideoxy-C. The products resulting from the reaction conducted on a "wild-type" allele template (wherein codon 72 encodes an arginine) and from the reaction conducted on a polymorphic  
 25 R72P allele template (wherein codon 72 encodes a proline) are shown below and designated as Cod72 G Arg (wt) and Cod72 C Pro, respectively. The masses for each product as can be measured by MALDI-TOF mass spectrometry are also provided (i.e., 5734.8 Da for the wild-type product and 5405.6 Da for the polymorphic product).

- In the PROBE analysis of the lipoprotein lipase gene polymorphic site, the extension reaction was performed using a mixture of ddA and ddT. The products resulting from the reaction conducted on a "wild-type" allele template (wherein codon 291 encodes an asparagine) and from the reaction conducted on a polymorphic N291S allele template (wherein codon 291 encodes a serine) are shown below and designated as 291Asn and 291Ser, respectively. The masses for each product as can be measured by MALDI-TOF mass spectrometry are also provided (i.e., 6438.2 Da for the wild-type product and 6758.4 Da for the polymorphic product).

**P53-1 (R72P)****PCR Product length: 407 bp (SEQ ID NO: 1)**

- US4-p53-ex4-F  
 15 ctctttttcac ccatctacag tcccccttgc cgtcccaagc ctg aggacotggt cctctgactg  
 ccccgagcga tattgaacaa tggttcactg aagaccagg tccagatgaa gctcccagaa  
 P53/72 72R  
 tgcacagagc tgcctcccgc gtggccctg caccagcagc tctacacgc ggggccctg  
 c 72P  
 20 caccagcccc ctctctggccc ctgtcatctt ctgtcccttc ccagaaaacc taccaggcca  
 gctacggttt ccgtctgggc ttcttgcaat ctgggacagc caagtctgtg acttgacagg  
 tcagttgccc tgaggggctg gcttccatga gactcaca  
 US5-p53/2-4R

**Primers (SEQ ID NOs: 2-4)**

- 25 p53-ex4FUS4 ccc agt cac gac gtt gta aaa cgc tga gga cct ggt cct ctg ac  
 US5P53/4R agc gga taa caa ttt cac aca ggt tga agt ctc atg gaa gcc  
 P53/72 gcc aga ggc tgc tcc cc

**Masses**

Allele	Product Termination: ddC	SEQ #	Length	Mass
P53/72	gccagaggctgctcccc	5	17	5132.4
Cod72 G Arg (wt)	gccagaggctgctccccgc	6	19	5734.8
Cod72 C Pro	gccagaggctgctccccc	7	18	5405.6

Biotinylated US5 primer is used in the PCR amplification.

**LPL-1 (N291S)**

Amino acid exchange asparagine to serine at codon 291 of the lipoprotein lipase gene.

**PCR Product length: 251 bp (SEQ ID NO: 15)****US4-LPL-F2 (SEQ ID NO: 16)**

5 gcgctccatt catctcttcca tcgactctct gttgaatgaa gaaaatccaa gtaaggccta  
 cagggtgcagt tccaaggaag cctttgagaa agggctctgc ttgagttgta gaaagaaccg  
 LPL-2 291N  
 ctgcaacaat ctgggctatg agatcaataa agtcagagcc aaaagaagca gcaaaatgta  
 cctgaagact cgttctcaga tgccc g 291S  
 US4-LPL-R2

**10 Primers (SEQ ID NOs: 16-18):**

US4-LPL-F2 ccc agt cac gac gtt gta aaa cgc ccc att cat ctc ttc

US5-LPL-R2 agc gga taa caa ttt cac aca ggc ggc atc tga gaa cga gtc

LPL-2 caa tct ggc cta tga gat ca

**Masses**

15 Allele	Product Termination: ddA, ddT	SEQ #	Length	Mass
LPL-2	caatctgggctatgagatca	19	20	6141
291 Asn	caatctgggctatgagatcaa	20	21	6438.2
291 Ser	caatctgggctatgagatcagt	21	22	6758.4

**20 Biotinylated US5 primer is used in the PCR amplification.****P21-1 (S31R)**

Amino acid exchange serine to arginine at codon 31 of the tumor suppressor gene p21. Product length: 207 bp (SEQ ID NO: 8)

US4p21c31-2F

25 gtcc gtcagaaccc atgcggcagc  
 p21/31-3 31S  
 aaggctcgcc gccgcctctt cggcccagtg gacagcgagc agctgagccg cgactgtgat  
 a 31R  
 30 gcgctaattg cgggctgcat ccaggaggcc cgtgagcgat ggaacttcga ctttgtcacc  
gagacaccac tgagggg  
 US5p21-2R

**Primers (SEQ ID NOs: 9-11)**

US4p21c31-2F ccc agt cac gac gtt gta aaa cgc tcc gtc aga acc cat gcg g

US5p21-2R agc gga taa caa ttt cac aca ggc tcc agt ggt gtc tcg gtg ac

35 P21/31-3 cag cga gca gct gag

**Masses**

Allele	Product Termination: ddC	SEQ #	Length	Mass
p21/31-3	cagcgagcagctgag	12	15	4627
P21/31-3 Ser (wt)	cagcgagcagctgagc	13	16	4900.2
5 P21/31-3 Arg	cagcgagcagctgagac	14	17	5213.4

Biotinylated US5 primer is used in the PCR amplification.

Each of the Caucasian subject DNA samples was individually analyzed by MALDI-TOF mass spectrometry to determine the identity of the nucleotide at the polymorphic sites. The genotypic results of each assay can be entered into the database. The results were then sorted according to age and/or sex to determine the distribution of allelic frequencies by age and/or sex. As depicted in the Figures showing histograms of the results, in each case, there was a differential distribution of the allelic frequencies of the genetic markers for the p21, p53 and lipoprotein lipase gene polymorphisms.

Figure 8 shows the results of the p21 genetic marker assays reveals a statistically significant decrease (from 13.3% to 9.2%) in the frequency of the heterozygous genotype (S31R) in Caucasians with age (18-49 years of age compared to 50-79 years of age). The frequencies of the homozygous (S31 and R31) genotypes for the two age groups are also shown, as are the overall frequencies of the S31 and R31 alleles in the two age groups (designated as \*S31 and \*R31, respectively in the Figure).

Figures 7A-C shows the results of the p53 genetic marker assays and reveals a statistically significant decrease (from 6.7% to 3.7%) in the frequency of the homozygous polymorphic genotype (P72) in Caucasians with age (18-59 years of age compared to 60-79 years of age). The frequencies of the homozygous "wild-type" genotype (R72) and the heterozygous genotype (R72P) for the two age groups are also shown, as are the overall frequencies of the R72 and P72 alleles in the two age

groups (designated as \*R72 and \*P72, respectively in the Figure). These results are consistent with the observation that allele is not benign, as p53 regulates expression of a second protein, p21, which inhibits cyclin-dependent kinases (CDKs) needed to drive cells through the cell-cycle (a mutation in either gene can disrupt the cell cycle leading to increased cell division).

Figure 2C shows the results of the lipoprotein lipase gene genetic marker assays reveals a statistically significant decrease (from 1.97% to 0.54%) in the frequency of the polymorphic allele (S291) in Caucasian males with age (see also Reymer *et al.* (1995) *Nature Genetics* 10:28-34). The frequencies of this allele in Caucasian females of different age groups are also shown.

## EXAMPLE 2

This example describes the use of MALDI-TOF mass spectrometry to analyze DNA samples of a number of subjects as individual samples and as pooled samples of multiple subjects to assess the presence or absence of a polymorphic allele (the 353Q allele) of the Factor VII gene and determine the frequency of the allele in the group of subjects. The results of this study show that essentially the same allelic frequency can be obtained by analyzing pooled DNA samples as by analyzing each sample separately and thereby demonstrate the quantitative nature of MALDI-TOF mass spectrometry in the analysis of nucleic acids.

### Factor VII

Factor VII is a serine protease involved in the extrinsic blood coagulation cascade. This factor is activated by thrombin and works with tissue factor (Factor III) in the processing of Factor X to Factor Xa. There is evidence that supports an association between polymorphisms in the Factor VII gene and increased Factor VII activity which can result in an elevated risk of ischemic cardiovascular disease, including myocardial infarction. The polymorphism investigated in this study is R353Q (i.e., a

substitution of a glutamic acid residue for an arginine residue at codon 353 of the Factor VII gene) (see Table 5).

**Analysis of DNA samples for the presence or absence of the 353Q allele of the Factor VII gene**

5

Genomic DNA was isolated from separate blood samples obtained from a large number of subjects divided into multiple groups of 92 subjects per group. Each sample of genomic DNA was analyzed using the BiomassPROBE™ assay as described in Example 1 to determine the presence or absence of the 353Q polymorphism of the Factor VII gene.

First, DNA from each sample was amplified in a polymerase chain reaction using primers F7-353FUS4 (SEQ ID NO: 24) and F7-353RUS5 (SEQ ID NO: 26) as shown below and using standard conditions, for example, as described in Example 1. One of the primers was biotinylated to permit immobilization of the amplification product to a solid support. The purified amplification products were immobilized via a biotin-avidin linkage to streptavidin-coated beads and the double-stranded DNA was denatured. A detection primer was then annealed to the immobilized DNA using conditions such as, for example, described in Example 1. The detection primer is shown as F7-353-P (SEQ ID NO: 27) below. The PROBE extension reaction was carried out using conditions, for example, such as those described in Example 1. The reaction was performed using ddG.

The DNA was denatured to release the extended primers from the immobilized template. Each of the resulting extension products was separately analyzed by MALDI-TOF mass spectrometry. A matrix such as 3-hydroxypicolinic acid (3-HPA) and a UV laser could be used in the MALDI-TOF mass spectrometric analysis. The products resulting from the reaction conducted on a "wild-type" allele template (wherein codon 353 encodes an arginine) and from the reaction conducted on a polymorphic 353Q allele template (wherein codon 353 encodes a glutamic acid) are

shown below and designated as 353 CGG and 353 CAG, respectively. The masses for each product as can be measured by MALDI-TOF mass spectrometry are also provided (i.e., 5646.8 Da for the wild-type product and 5960 Da for the polymorphic product).

5        The MALDI-TOF mass spectrometric analyses of the PROBE reactions of each DNA sample were first conducted separately on each sample (250 nanograms total concentration of DNA per analysis). The allelic frequency of the 353Q polymorphism in the group of 92 subjects was calculated based on the number of individual subjects in which it was  
10 detected.

Next, the samples from 92 subjects were pooled (250 nanograms total concentration of DNA in which the concentration of any individual DNA is 2.7 nanograms) and the pool of DNA was subjected to MALDI-TOF mass spectrometric analysis. The area under the signal  
15 corresponding to the mass of the 353Q polymorphism PROBE extension product in the resulting spectrum was integrated in order to quantitate the amount of DNA present. The ratio of this amount to total DNA was used to determine the allelic frequency of the 353Q polymorphism in the group of subjects. This type of individual sample vs. pooled sample analysis  
20 was repeated for numerous different groups of 92 different samples.

The frequencies calculated based on individual MALDI-TOF mass spectrometric analysis of the 92 separate samples of each group of 92 are compared to those calculated based on MALDI-TOF mass spectrometric analysis of pools of DNA from 92 samples in Figure 9.  
25 These comparisons are shown as "pairs" of bar graphs in the Figure, each pair being labeled as a separate "pool" number, e.g., P1, P16, P2, etc. Thus, for example, for P1, the allelic frequency of the polymorphism calculated by separate analysis of each of the 92 samples was 11.41% and the frequency calculated by analysis of a pool of all of the 92 DNA  
30 samples was 12.09%.

- The similarity in frequencies calculated by analyzing separate DNA samples individually and by pooling the DNA samples demonstrates that it is possible, through the quantitative nature of MALDI-TOF mass spectrometry, to analyze pooled samples and obtain accurate frequency determinations. The ability to analyze pooled DNA samples significantly reduces the time and costs involved in the use of the non-selected, healthy databases as described herein. It has also been shown that it is possible to decrease the DNA concentration of the individual samples in a pooled mixture from 2.7 nanograms to 0.27 nanograms without any change in the quality of the spectrum or the ability to quantitate the amount of sample detected.

#### Factor VII R353Q PROBE Assay

PROBE Assay for cod353 CGG>CAG (Arg>Gln), Exon 9 G>A.

**PCR fragment: 134 bp (incl. US tags; SEQ ID Nos. 22 and 23)**

- Frequency of A allele: Europeans about 0.1, Japanese/Chinese about 0.03-0.05 (Thromb. Haemost. 1995, 73:617-22; Diabetologia 1998, 41:760-6):

#### F7-353FUS4>

- 1201 GTGCCGGCTA CTCGGATGGC AGCAAGGACT CCTGCAAGGG GGACAGTGGAGGCCCCATCG  
F7-353-P> A <F7-353RUS5  
1261 CCACCCACTA CCGGGGCACG TGGTACCTGA CGGGCATCGT CAGCTGGGGC  
CAGGGCTCGC

- Primers (SEQ ID NOS: 24-26) Tm<sup>°</sup>  
F7-353FUS4 CCC AGT CAC GAC GTT GTA AAA CGA TGG CAG CAA GGA CTC CTG 64°C  
F7-353-P CAC ATG CCA CCC ACT ACC  
F7-353RUS5 AGC GGA TAA CAA TTT CAC ACA GGT GAC GAT GCC CGT CAG GTA C 64°C

#### Masses

- | Allele       | Product Termination: ddG | SEQ # | Length | Mass   |
|--------------|--------------------------|-------|--------|--------|
| F7-353-P     | atgccacccactacc          | 27    | 18     | 5333.6 |
| 353 CGG      | cacatgccacccactaccg      | 28    | 19     | 5646.8 |
| 353 CAG      | cacatgccacccactaccag     | 29    | 20     | 5960   |
| US5-bio bio- | agcggataacaatttcacacagg  | 30    | 23     | 7648.6 |

## Conclusion

The above examples demonstrate an effect of altered frequency of disease causing genetic factors within the general population. Interpretation of those results allows prediction of the medical relevance of polymorphic genetic alterations. In addition, conclusions can be drawn with regard to their penetrance, diagnostic specificity, positive predictive value, onset of disease, most appropriate onset of preventive strategies, and the general applicability of genetic alterations identified in isolated populations to panmixed populations. Therefore, an age- and sex-stratified population-based sample bank that is ethnically homogenous is a suitable tool for rapid identification and validation of genetic factors regarding their potential medical utility.

## EXAMPLE 3

### MORBIDITY AND MORTALITY MARKERS

#### 15 Sample Band and Initial Screening

Healthy samples were obtained through the blood bank of San Bernardino, CA. Donors signed prior to the blood collection a consent form and agreed that their blood will be used in genetic studies with regard to human aging. All samples were anonymized. Tracking back of samples is not possible.

#### Isolation of DNA from blood samples of a healthy donor population

Blood is obtained from a donor by venous puncture and preserved with 1mM EDTA pH 8.0. Ten milliliters of whole blood from each donor was centrifuged at 2000x g. One milliliter of the buffy coat was added to 9 milliliters of 155mM  $\text{NH}_4\text{Cl}$ , 10mM  $\text{KHCO}_3$ , and 0.1mM  $\text{Na}_2\text{EDTA}$ , incubated 10 minutes at room temperature and centrifuged for 10 minutes at 2000x g. The supernatant was removed, and the white cell pellet was washed in 155mM  $\text{NH}_4\text{Cl}$ , 10mM  $\text{KHCO}_3$ , and 0.1mM  $\text{Na}_2\text{EDTA}$  and resuspended in 4.5 milliliters of 50mM Tris, 5mM EDTA, and 1% SDS. Proteins were precipitated from the cell lysate by 6M

Ammonium Acetate, pH 7.3, and separated from the nucleic acid by centrifugation 3000x g. The nucleic acid was recovered from the supernatant by the addition of an equal volume of 100% isopropanol and centrifugation at 2000x g. The dried nucleic acid pellet was hydrated in

- 5 10mM Tris pH 7.6 and 1mM Na<sub>2</sub>EDTA and stored at 4C.

In this study, samples were pooled as shown in Table 1. Both parents of the blood donors were of Caucasian origin.

Table 1

Pool ID	Sex	Age-range	# individuals
SP1	Female	18-39 years	276
SP2	Males	18-39 years	276
SP3	Females	60-69 years	184
SP4	Males	60-79 years	368

- 15 More than 400 SNPs were tested using all four pools. After one test run 34 assays were selected to be re-assayed at least once. Finally, 10 assays showed repeatedly differences in allele frequencies of several percent and, therefore, fulfilled the criteria to be tested using the individual samples. Average allele frequency and standard deviation is tabulated in Table 2.
- 20

Table 2

Assay ID	SP1	SP1-STD	SP2	SP2-STD	SP3	SP3-STD	SP4	SP4-STD
47861	0.457	0.028	0.433	0.042	0.384	0.034	0.000	0.015
47751	0.276	0.007	0.483	0.006	0.428	0.052	0.400	0.097
48319	0.676	0.013	0.627	0.018	0.755	0.006	0.006	0.034
48070	0.581	0.034	0.617	0.045	0.581	n.a.	0.539	0.032
49807	0.504	0.034	0.422	0.020	0.477	0.030	0.556	0.005

49534	0.537	0.017	0.503	n.a.	0.623	0.023	0.535	0.009
49733	0.560	0.006	0.527	0.059	0.546	0.032	0.436	0.016
49947	0.754	0.008	0.763	0.047	0.736	0.052	0.689	0.025
50128	0.401	0.022	0.363	0.001	0.294	0.059	0.345	0.013
63306	0.697	0.012	0.674	0.013	0.712	0.017	0.719	0.005

So far, 7 out of the 10 potential morbidity markers were fully analyzed. Additional information about genes in which these SNPs are located was gathered through publicly databases like Genbank.

#### 10 AKAPS

- Candidate morbidity and mortality markers include housekeeping genes, such as genes involved in signal transduction. Among such genes are the A-kinase anchoring proteins (AKAPs) genes, which participate in signal transduction pathways involving protein phosphorylation. Protein phosphorylation is an important mechanism for enzyme regulation and the transduction of extracellular signals across the cell membrane in eukaryotic cells. A wide variety of cellular substrates, including enzymes, membrane receptors, ion channels and transcription factors, can be phosphorylated in response to extracellular signals that interact with cells.
- A key enzyme in the phosphorylation of cellular proteins in response to hormones and neurotransmitters is cyclic AMP (cAMP)-dependent protein kinase (PKA). Upon activation by cAMP, PKA thus mediates a variety of cellular responses to such extracellular signals. An array of PKA isozymes are expressed in mammalian cells. The PKAs usually exist as inactive tetramers containing a regulatory (R) subunit dimer and two catalytic (C) subunits. Genes encoding three C subunits ( $C\alpha$ ,  $C\beta$  and  $C\gamma$ ) and four R subunits ( $R1\alpha$ ,  $R1\beta$ ,  $R1\alpha$  and  $R1\beta$ ) have been identified [see Takio *et al.* (1982) *Proc. Natl. Acad. Sci. U.S. A.* 79:2544-2548; Lee *et al.* (1983)

*Proc. Natl. Acad. Sci. U.S. A.* 80:3608-3612; Jahnsen *et al.* (1996) *J. Biol. Chem.* 261:12352-12361; Clegg *et al.* (1988) *Proc. Natl. Acad. Sci. U.S. A.* 85:3703-3707; and Scott (1991) *Pharmacol. Ther.* 50:123-145]. The type I (RI)  $\alpha$  and type II (RII)  $\alpha$  subunits are distributed ubiquitously, whereas RI $\beta$  and RII $\beta$  are present mainly in brain [see, *e.g.*, Miki and Eddy (1999) *J. Biol. Chem.* 274:29057-29062]. The type I PKA holoenzyme (RI $\alpha$  and RI $\beta$ ) is predominantly cytoplasmic, whereas the majority of type II PKA (RII $\alpha$  and RII $\beta$ ) associates with cellular structures and organelles [Scott (1991) *Pharmacol. Ther.* 50:123-145]. Many hormones and other signals act through receptors to generate cAMP which binds to the R subunits of PKA and releases and activates the C subunits to phosphorylate proteins. Because protein kinases and their substrates are widely distributed throughout cells, there are mechanisms in place in cells to localize protein kinase-mediated responses to different signals. One such mechanism involves subcellular targeting of PKAs through association with anchoring proteins, referred to as A-kinase anchoring proteins (AKAPs), that place PKAs in close proximity to specific organelles or cytoskeletal components and particular substrates thereby providing for more specific PKA interactions and localized responses [see, *e.g.*, Scott *et al.* (1990) *J. Biol. Chem.* 265:21561-21566; Bregman *et al.* (1991) *J. Biol. Chem.* 266:7207-7213; and Miki and Eddy (1999) *J. Biol. Chem.* 274:29057-29062]. Anchoring not only places the kinase close to preferred substrates, but also positions the PKA holoenzyme at sites where it can optimally respond to fluctuations in the second messenger cAMP [Mochly-Rosen (1995) *Science* 268:247-251; Faux and Scott (1996) *Trends Biochem. Sci.* 21:312-315; Hubbard and Cohen (1993) *Trends Biochem. Sci.* 18:172-177].

Up to 75% of type II PKA is localized to various intracellular sites through association of the regulatory subunit (RII) with AKAPs [see, *e.g.*, Hausken *et al.* (1996) *J. Biol. Chem.* 271:29016-29022]. RII subunits of

PKA bind to AKAPs with nanomolar affinity [Carr *et al.* (1992) *J. Biol. Chem.* 267:13376-13382], and many AKAP-RII complexes have been isolated from cell extracts. RI subunits of PKA bind to AKAPs with only micromolar affinity [Burton *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:11067-11072]. Evidence of binding of a PKA RI subunit to an AKAP has been reported [Miki and Eddy (1998) *J. Biol. Chem.* 273:34384-34390] in which RI $\alpha$ -specific and RI $\alpha$ /RII $\alpha$  dual specificity PKA anchoring domains were identified on FSC1/AKAP82. Additional dual specific AKAPs, referred to as D-AKAP1 and D-AKAP2, which interact with the type I and type II regulatory subunits of PKA have also been reported [Huang *et al.* (1997) *J. Biol. Chem.* 272:8057-8064; Huang *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:11184-11189].

More than 20 AKAPs have been reported in different tissues and species. Complementary DNAs (cDNAs) encoding AKAPs have been isolated from diverse species, ranging from *Caenorhabditis elegans* and *Drosophila* to human [see, *e.g.*, Colledge and Scott (1999) *Trends Cell Biol.* 9:216-221]. Regions within AKAPs that mediate association with RII subunits of PKA have been identified. These regions of approximately 10-18 amino acid residues vary substantially in primary sequence, but secondary structure predictions indicate that they are likely to form an amphipathic helix with hydrophobic residues aligned along one face of the helix and charged residues along the other [Carr *et al.* (1991) *J. Biol. Chem.* 266:14188-14192; Carr *et al.* (1992) *J. Biol. Chem.* 267:13376-13382]. Hydrophobic amino acids with a long aliphatic side chain, *e.g.*, valine, leucine or isoleucine, may participate in binding to RII subunits [Glantz *et al.* (1993) *J. Biol. Chem.* 268:12796-12804].

Many AKAPs also have the ability to bind to multiple proteins, including other signaling enzymes. For example, AKAP79 binds to PKA, protein kinase C (PKC) and the protein phosphatase calcineurin (PP2B) [Coghlan *et al.* (1995) *Science* 267:108-112 and Klauk *et al.* (1996)

*Science* 271:1589-1592]. Therefore, the targeting of AKAP79 to neuronal postsynaptic membranes brings together enzymes with opposite catalytic activities in a single complex.

- AKAPs thus serve as potential regulatory mechanisms that increase the selectivity and intensity of a cAMP-mediated response. There is a need, therefore, to identify and elucidate the structural and functional properties of AKAPs in order to gain a complete understanding of the important role these proteins play in the basic functioning of cells.

#### **AKAP10**

- 10 The sequence of a human AKAP10 cDNA (also referred to as D-AKAP2) is available in the GenBank database, at accession numbers AF037439 (SEQ ID NO: 31) and NM 007202. The AKAP10 gene is located on chromosome 17.

- The sequence of a mouse D-AKAP2 cDNA is also available in the GenBank database (see accession number AF021833). The mouse D-AKAP2 protein contains an RGS domain near the amino terminus that is characteristic of proteins that interact with G $\alpha$  subunits and possess GTPase activating protein-like activity [Huang *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:11184-11189]. The human AKAP10 protein also has sequences homologous to RGS domains. The carboxy-terminal 40 residues of the mouse D-AKAP2 protein are responsible for the interaction with the regulatory subunits of PKA. This sequence is fairly well conserved between the mouse D-AKAP2 and human AKAP10 proteins.

#### **Polymorphisms of the human AKAP10 gene and polymorphic AKAP10 proteins**

- Polymorphisms of AKAP genes that alter gene expression, regulation, protein structure and/or protein function are more likely to have a significant effect on the regulation of enzyme (particularly PKA) activity, cellular transduction of signals and responses thereto and on the basic functioning of cells than polymorphisms that do not alter gene

and/or protein function. Included in the polymorphic AKAPs provided herein are human AKAP10 proteins containing differing amino acid residues at position number 646.

- 5 Amino acid 646 of the human AKAP10 protein is located in the carboxy-terminal region of the protein within a segment that participates in the binding of R-subunits of PKAs. This segment includes the carboxy-terminal 40 amino acids.

- 10 The amino acid residue reported for position 646 of the human AKAP10 protein is an isoleucine. Polymorphic human AKAP10 proteins provided herein have the amino acid sequence but contain residues other than isoleucine at amino acid position 646 of the protein. In particular embodiments of the polymorphic human AKAP10 proteins provided herein, the amino acid at position 646 is a valine, leucine or phenylalanine residue.

- 15 **An A to G transition at nucleotide 2073 of the human AKAP10 coding sequence**

- As described herein, an allele of the human AKAP10 gene that contains a specific polymorphism at position 2073 of the coding sequence and thereby encodes a valine at position 646 has been detected  
20 in varying frequencies in DNA samples from younger and older segments of the human population. In this allele, the A at position 2073 of the AKAP10 gene coding sequence is changed from an A to a G, giving rise to an altered sequence in which the codon for amino acid 646 changes from ATT, coding for isoleucine, to GTT, coding for valine.

- 25 **Morbidity marker 1: human protein kinase A anchoring protein (AKAP10-1)**

PCR Amplification and BiomassPROBE assay detection of AKAP10-1 in a healthy donor population

PCR Amplification of donor population for AKAP 10

- 30 PCR primers were synthesized by OPERON using phosphoramidite chemistry. Amplification of the AKAP10 target sequence was carried out

- in single 50 $\mu$ l PCR reaction with 100ng-1ug of pooled human genomic DNAs in a 50 $\mu$ l PCR reaction. Individual DNA concentrations within the pooled samples were present in equal concentration with the final concentration ranging from 1-25ng. Each reaction containing IX PCR
- 5 buffer (Qiagen, Valencia, CA), 200uM dNTPs, 1U Hotstar Taq polymerase (Qiagen, Valencia, CA), 4mM MgCl<sub>2</sub>, and 25pmol of the forward primer containing the universal primer sequence and the target specific sequence 5'-TCTCAATCATGTGCATTGAGG-3'(SEQ ID NO: 45), 2pmol of the reverse primer
- 10 5'-AGCGGATAACAATTTACACAGGGATCACACAGCCATCAGCAG-3' (SEQ ID NO: 46), and 10pmol of a biotinylated universal primer complementary to the 5' end of the PCR amplicon 5'-AGCGGATAACAATTTACACAGG-3'(SEQ ID NO: 47). After an initial round of amplification with the target with the specific forward and
- 15 reverse primer, the 5' biotinylated universal primer then hybridized and acted as a reverse primer thereby introducing a 3' biotin capture moiety into the molecule. The amplification protocol results in a 5'-biotinylated double stranded DNA amplicon and dramatically reduces the cost of high throughput genotyping by eliminating the need to 5' biotin label each
- 20 forward primer used in a genotyping. Thermal cycling was performed in 0.2mL tubes or 96 well plate using an MJ Research Thermal Cycler (calculated temperature) with the following cycling parameters: 94° C for 5 min; 45 cycles: 94° C for 20 sec, 56° C for 30 sec, 72° C for 60 sec; 72° C 3min.
- 25 Immobilization of DNA
- The 50 $\mu$ l PCR reaction was added to 25ul of streptavidin coated magnetic bead (Dyna) prewashed three times and resuspended in 1M NH<sub>4</sub>Cl, 0.06M NH<sub>4</sub>OH. The PCR amplicons were allowed to bind to the beads for 15 minutes at room temperature. The beads were then collected with a
- 30 magnet and the supernatant containing unbound DNA was removed. The

unbound strand was release from the double stranded amplicons by incubation in 100mM NaOH and washing of the beads three times with 10mM Tris pH 8.0.

BiomassPROBE assay analysis of donor population for AKAP10-1 (clone 48319)

- Genotyping using the BiomassPROBE assay methods was carried out by resuspending the DNA coated magnetic beads in 26mM Tris-HCl pH 9.5, 6.5 mM MgCl<sub>2</sub> and 50mM each of dTTP and 50mM each of ddCTP, ddATP, ddGTP, 2.5U of a thermostable DNA polymerase (Amersham) and 20pmol of a template specific oligonucleotide PROBE primer 5'-CTGGCGCCACGTGGTCAA-3' (SEQ ID NO: 48) (Operon). Primer extension occurs with three cycles of oligonucleotide primer hybridization and extension. The extension products were analyzed after denaturation from the template with 50mM NH<sub>4</sub>Cl and transfer of 150nL each sample to a silicon chip preloaded with 150nL of H3PA matrix material. The sample material was allowed to crystallize and was analyzed by MALDI-TOF (Bruker, PerSeptive). The SNP that is present in AKAP10-1 is a T to C transversion at nucleotide number 156277 of the sequence of a genomic clone of the AKAP10 gene (GenBank Accession No. AC005730) (SEQ ID NO: 36). SEQ ID NO: 35: represents the nucleotide sequence of human chromosome 17, which contains the genomic nucleotide sequence of the human AKAP10 gene, and SEQ ID NO: represents the nucleotide sequence of human chromosome 17, which contains the genomic nucleotide sequence of the human AKAP10-1 allele. The mass of the primer used in the BioMass probe reaction was 5500.6 daltons. In the presence of the SNP, the primer is extended by the addition of ddC, which has a mass of 5773.8. The wildtype gene results in the addition of dT and ddG to the primer to produce an extension product having a mass of 6101 daltons.

The frequency of the SNP was measured in a population of age selected healthy individuals. Five hundred fifty-two (552) individuals between the ages of 18-39 years (276 females, 276 males) and 552 individuals between the ages of 60-79 (184 females between the ages of 60-69, 368 males between the age of 60-79) were tested for the presence of the polymorphism localized in the non-translated 3'-region of AKAP 10. Differences in the frequency of this polymorphism with increasing age groups were observed among healthy individuals. Statistical analysis showed that the significance level for differences in the allelic frequency for alleles between the "younger" and the "older" populations was  $p=0.0009$  and for genotypes was  $p=0.003$ . Differences between age groups are significant. For the total population allele significance is  $p=0.0009$ , and genotype significance is  $p=0.003$ .

This marker led to the best significant result with regard to allele and genotype frequencies in the age-stratified population. Figure 19 shows the allele and genotype frequency in both genders as well as in the entire population. For latter the significance for alleles was  $p=0.0009$  and for genotypes was  $p=0.003$ . The young and old populations were in Hardy-Weinberg equilibrium. A preferential change of one particular genotype was not seen.

The polymorphism is localized in the non-translated 3'-region of the gene encoding the human protein kinase A anchoring protein (AKAP10). The gene is located on chromosome 17. Its structure includes 15 exons and 14 intervening sequences (introns). The encoded protein is responsible for the sub-cellular localization of the cAMP-dependent protein kinase and, therefore, plays a key role in the G-protein mediated receptor-signaling pathway (Huang et al. PNAS (1007) 94:11184-11189). Since its localization is outside the coding region, this polymorphism is most likely in linkage disequilibrium (LD) with other non-synonymous polymorphisms that could cause amino acid substitutions and

subsequently alter the function of the protein. Sequence comparison of different Genbank database entries concerning this gene revealed further six potential polymorphisms of which two are supposed to change the respective amino acid (see Table 3).

5 Table 3

Exon	Codon	Nucleotides	Amino acid
3	100	GCT > GCC	Ala > Ala
4	177	AGT > GTG	Met > Val
3	424	GGG > GGC	Gly > Gly
10 10	524	CCG > CTG	Pro > Leu
12	591	GTG > GTC	Val > Val
12	599	CGC > CGA	Arg > Arg

15 **Morbidity marker 2: human protein kinase A anchoring protein (AKAP10-5)**

**Discovery of AKAP10-5 Allele (SEQ ID NO: 33)**

Genomic DNA was isolated from blood (as described above) of seventeen (17) individuals with a genotype CC at the AKAP10-1 gene locus and a single heterozygous individual (CT) (as described). A target sequence in the AKAP10-1 gene which encodes the C-terminal PKA binding domain was amplified using the polymerase chain reaction. PCR primers were synthesized by OPERON using phosphoramidite chemistry. Amplification of the AKAP10-1 target sequence was carried out in individual 50 $\mu$ l PCR reaction with 25ng of human genomic DNA templates. Each reaction containing 1 X PCR buffer (Qiagen, Valencia, CA), 200 $\mu$ M dNTPs, 1U Hotstar Taq polymerase (Qiagen, Valencia, CA), 4mM MgCl<sub>2</sub>, 25pmol of the forward primer (Ex13F) containing the universal primer sequence and the target specific sequence 5'-TCC CAA AGT GCT GGA ATT AC-3' (SEQ ID NO: 53), and 2pmol of the reverse primer (Ex14R) 5'-GTC CAA TAT ATG CAA ACA GTT G-3' (SEQ ID NO:

- 54). Thermal cycling was performed in 0.2mL tubes or 96 well plate using an MJ Research Thermal Cycler (MJ Research, Waltham, MA) (calculated temperature) with the following cycling parameters: 94° C for 5 min; 45 cycles; 94° C for 20 sec, 56° C for 30 sec, 72° C for 60 sec; 5 72° C 3min. After amplification the amplicons were purified using a chromatography (Mo Bio Laboratories (Solana Beach, CA)).

- The sequence of the 18 amplicons, representing the target region, was determined using a standard Sanger cycle sequencing method with 25nmol of the PCR amplicon, 3.2uM DNA sequencing primer 5'-CCC ACA 10 GCA GTT AAT CCT TC-3'(SEQ ID NO: 55), and chain terminating dRhodamine labeled 2', 3' dideoxynucleotides (PE Biosystems, Foster City, CA) using the following cycling parameters: 96° C for 15 seconds; 25 cycles: 55° C for 15 seconds, 60° C for 4 minutes. The sequencing products precipitated by 0.3M NaOAc and ethanol. The precipitate was 15 centrifuged and dried. The pellets were resuspended in deionized formamide and separated on a 5% polyacrylimide gel. The sequence was determined using the "Sequencher" software (Gene Codes, Ann Arbor, MI).

- The sequence of all 17 of the amplicons, which are homozygous 20 for the AKAP10-1 SNP of the amplicons, revealed a polymorphism at nucleotide position 152171 (numbering for GenBank Accession No. AC005730 for AKAP10 genomic clone (SEQ ID NO: 35)) with A replaced by G. This SNP can also be designated as located at nucleotide 2073 of a cDNA clone of the wildtype AKAP10 (GenBank Accession No. 25 AF037439) (SEQ ID NO: 31). The amino acid sequence of the human AKAP10 protein is provided as SEQ ID NO: 32. This single nucleotide polymorphism was designated as AKAP10-5 (SEQ ID NO: 33) and resulted in a substitution of a valine for an isoleucine residue at amino acid position 646 of the amino acid sequence of human AKAP10 (SEQ ID 30 NO: 32).

### PCR Amplification and BiomassPROBE assay detection of AKAP10-5 in a healthy donor population

The healthy population stratified by age is a very efficient and a universal screening tool for morbidity associated genes by allowing for the  
5 detection of changes of allelic frequencies in the young compared to the old population. Individual samples of this healthy population base can be pooled to further increase the throughput.

Healthy samples were obtained through the blood bank of San Bernardino, CA. Both parents of the blood donors were of Caucasian  
10 origin. Practically a healthy subject, when human, is defined as human donor who passes blood bank criteria to donate blood for eventual use in the general population. These criteria are as follows: free of detectable viral, bacterial, mycoplasma, and parasitic infections; not anemic; and then further selected based upon a questionnaire regarding history (see  
15 Figure 3). Thus, a healthy population represents an unbiased population of sufficient health to donate blood according to blood bank criteria, and not further selected for any disease state. Typically such individuals are not taking any medications.

PCR primers were synthesized by OPERON using phosphoramidite  
20 chemistry. Amplification of the AKAP10 target sequence was carried out in a single 50 $\mu$ l PCR reaction with 100ng- 1 $\mu$ g of pooled human genomic DNAs in a 50 $\mu$ l PCR reaction. Individual DNA concentrations within the pooled samples were present in equal concentration with the final concentration ranging from 1-25ng. Each reaction contained 1X PCR  
25 buffer (Qiagen, Valencia, CA), 200 $\mu$ M dNTPs, 1U Hotstar Taq polymerase (Qiagen, Valencia, CA), 4mM MgCl<sub>2</sub>, and 25pmol of the forward primer containing the universal primer sequence and the target specific sequence 5'-AGCGGATAACAATTTACACAGGGAGCTAGCTTGGAAGAT  
TGC-3' (SEQ ID NO: 41), 2pmol of the reverse primer  
30 5'-GTCCAATATATGCAAACAGTTG-3' (SEQ ID NO: 54), and 10pmol of a

biotinylated universal primer complementary to the 5' end of the PCR amplicon BIO:5'-AGCGGATAACAATTTTCACACAGG-3' (SEQ ID NO: 43). After an initial round of amplification with the target with the specific forward and reverse primer, the 5' biotinylated universal primer can then be hybridized and acted as a forward primer thereby introducing a 5' biotin capture moiety into the molecule. The amplification protocol resulted in a 5'-biotinylated double stranded DNA amplicon and dramatically reduced the cost of high throughput genotyping by eliminating the need to 5' biotin label every forward primer used in a genotyping.

Thermal cycling was performed in 0.2mL tubes or 96 well plate using an MJ Research Thermal Cycler (calculated temperature) with the following cycling parameters: 94° C for 5 min; 45 cycles: 94° C for 20 sec, 56° C for 30 sec; 72° C for 60 sec; 72° C 3min.

#### 15 **Immobilization of DNA**

The 50  $\mu$ l PCR reaction was added to 25 $\mu$ L of streptavidin coated magnetic beads (Dyna, Oslo, Norway), which were prewashed three times and resuspended in 1M NH<sub>4</sub>Cl, 0.06M NH<sub>4</sub>OH. The 5' end of one strand of the double stranded PCR amplicons were allowed to bind to the beads for 15 minutes at room temperature. The beads were then collected with a magnet and the supernatant containing unbound DNA was removed. The hybridized but unbound strand was released from the double stranded amplicons by incubation in 100mM NaOH and washing of the beads three times with 10mM Tris pH 8.0.

#### 25 **Detection of AKAP10-5 using BiomassPROBE™ Assay**

BiomassPROBE™ assay of primer extension analysis (see, U.S. Patent No. 6,043,031) of donor population for AKAP 10-5 (SEQ ID NO: 33) was performed. Genotyping using these methods was carried out by resuspending the DNA coated magnetic beads in 26mM Tris-HCL pH 9.5, 6.5 mM MgCl<sub>2</sub>, 50mM dTTP, 50mM each of ddCTP, ddATP, ddGTP, 2.5U

of a thermostable DNA polymerase (Amersham), and 20pmol of a template specific oligonucleotide PROBE primer

5'-ACTGAGCCTGCTGCATAA-3' (SEQ ID NO: 44) (Operon). Primer extension occurs with three cycles of oligonucleotide primer with

- 5 hybridization and extension. The extension products were analyzed after denaturation from the template with 50 mM NH<sub>4</sub>Cl and transfer of 150 nL of each sample to a silicon chip preloaded with 150 nL of H3PA matrix material. The sample material was allowed to crystallize and analyzed by MALDI-TOF (Bruker, PerSeptive). The primer has a mass of 5483.6  
10 daltons. The SNP results in the additional of a ddC to the primer, giving a mass of 5756.8 daltons for the extended product. The wild type results in the addition a T and ddG to the primer giving a mass of 6101 daltons.

The frequency of the SNP was measured in a population of age selected healthy individuals. Seven hundred thirteen (713) individuals  
15 under 40 years of age (360 females, 353 males) and 703 individuals over 60 years of age (322 females, 381 males) were tested for the presence of the SNP, AKAP10-5 (SEQ ID NO: 33). Results are presented below in Table 1.

20

25

30

TABLE 1					
AKAP10-5 (2073V) frequency comparison in 2 age groups					
			<40	>60	delta G allele
Female	Alleles	*G	38.6	34.6	4.0
		*A	61.4	65.4	
	Genotypes	G	13.9	11.8	2.1
		GA	49.4	45.7	
Male	Alleles	*G	41.4	37.0	4.4
		*A	58.6	63.0	
	Genotypes	G	18.4	10.8	7.7
		GA	49.4	45.7	

		GA	45.9	52.5	
		A	35.7	36.7	
<b>Total</b>	<b>Alleles</b>	<b>*G</b>	<b>40.0</b>	<b>35.9</b>	<b>4.1</b>
		<b>*A</b>	<b>60.0</b>	<b>64.1</b>	
	<b>Genotypes</b>	<b>G</b>	<b>16.1</b>	<b>11.2</b>	<b>4.9</b>
		<b>GA</b>	<b>47.7</b>	<b>49.4</b>	
		<b>A</b>	<b>36.2</b>	<b>39.4</b>	

Figure 20 graphically shows these results of allele and genotype distribution in the age and sex stratified Caucasian population.

**Morbidity marker 3: human methionine sulfoxide reductase A (msrA)**

The age-related allele and genotype frequency of this marker in both genders and the entire population is shown in Figure 21. The decrease of the homozygous CC genotype in the older male population is highly significant.

**Methionine sulfoxide reductase A (#63306)**

PCR Amplification and BiomassPROBE assay detection of the human methioine sulfoxid reductase A (h-msr-A) in a healthy donor population

**PCR Amplification of donor population for h-msr-A**

PCR primers were synthesized by OPERON using phosphoramidite chemistry. Amplification of the AKAP10 target sequence was carried out in single 50 $\mu$ l PCR reaction with 100ng-1 $\mu$ g of pooled human genomic DNA templates in a 50 $\mu$ l PCR reaction. Individual DNA concentrations within the pooled samples were present in an equal concentration with the final concentration ranging from 1-25ng. Each reaction containing 1 X PCR buffer (Qiagen, Valencia, CA), 200 $\mu$ M dNTPs, 1U Hotstar Taq polymerase (Qiagen, Valencia, CA), 4mM MgCl<sub>2</sub>, 25pmol of the forward primer containing the universal primer sequence and the target specific sequence 5'-TTTCTCTGCACAGAGAGGC-3' (SEQ ID NO: 49), 2pmol of

the reverse primer

5'-AGCGGATAACAATTTACACAGGGCTGAAATCCTTCGCTTTACC-3'  
(SEQ ID NO: 50), and 10pmol of a biotinylated universal primer  
complementary to the 5' end of the PCR amplicon

- 5 5'-AGCGGATAACAATTTACACAGG-3' (SEQ ID NO: 51). After an initial  
round of amplification of the target with the specific forward and reverse  
primers, the 5' biotinylated universal primer was then hybridized and  
acted as a reverse primer thereby introducing a 3' biotin capture moiety  
into the molecule. The amplification protocol results in a 5'-biotinylated  
10 double stranded DNA amplicon and and dramatically reduces the cost of  
high throughput genotyping by eliminating the need to 5' biotin label each  
forward primer used in a genotyping. Thermal cycling was performed in  
0.2mL tubes or 96 well plate using an MJ Research Thermal Cycler  
(calculated temperature) with the following cycling parameters: 94° C for  
15 5 min; 45 cycles: 94° C for 20 sec, 56° C for 30 sec, 72° C for 60 sec;  
72° C 3min.

#### Immobilization of DNA

- The 50μl PCR reaction was added to 25ul of streptavidin coated  
magnetic bead (Dyna) prewashed three times and resuspended in 1M  
20 NH<sub>4</sub>Cl, 0.06M NH<sub>4</sub>OH. The PCR amplicons were allowed to bind to the  
beads for 15 minutes at room temperature. The beads were then  
collected with a magnet and the supernatant containing unbound DNA  
was removed. The unbound strand was release from the double stranded  
amplicons by incubation in 100mM NaOH and washing of the beads three  
25 times with 10mM Tris pH 8.0.

#### **BiomassPROBE assay analysis of donor population for h-msr A**

- Genotyping using the BiomassPROBE assay methods was carried  
out by resuspending the he DNA coated magnetic beads in 26mM  
Tris-HCl pH 9.5, 6.5 mM MgCl<sub>2</sub>, 50mM of dTTPs and 50mM each of  
30 ddCTP, ddATP, ddGTP, 2.5U of a thermostable DNA polymerase

(Ambersham), and 20pmol of a template specific oligonucleotide PROBE primer 5'-CTGAAAAGGGAGAGAAAAG-3' (Operon) (SEQ ID NO: 52).

Primer extension occurs with three cycles of oligonucleotide primer with hybridization and extension. The extension products were analyzed after

- 5 denaturation from the template with 50mM  $\text{NH}_4\text{Cl}$  and transfer of 150nl each sample to a silicon chip preloaded with 150nl of H3PA matrix material. The sample material was allowed to crystallize and analyzed by MALDI-TOF (Bruker, PerSeptive). The SNP is represented as a T to C tranversion in the sequence of two ESTs. The wild type is represented by
- 10 having a T at position 128 of GenBank Accession No. AW 195104, which represents the nucleotide sequence of an EST which is a portion of the wild type human msrA gene (SEQ ID NO: 39). The SNP is presented as a C at position 129 of GenBank Accession No. AW 874187, which represents the nucleotide sequence of an EST which is a portion of an
- 15 allele of the human msrA gene (SEQ ID NO: 40 ).

- In a genomic sequence the SNP is represented as an A to G tranversion. The primer utilized in the BioMass probe reaction had a mass of 5654.8 daltons. In the presence of the SNP the primer is extended by the incorporation of a ddC and has a mass of 5928. In the
- 20 presence of the wildtype the primer is extended by adding a dT and a DDC to produce a mass of 6232.1 daltons.

- The frequency of the SNP was measured in a population of age selected healthy individuals. Five hundred fifty-two (552) individuals between the ages of 18-39 years (276 females, 276 males and 552
- 25 individuals between the age of 60-79 (184 females between the ages of 60-69, 368 males between the age of 60-79) were tested for the presence of the polymorphism localized in the nontranslated 3'region of h-msr-A.

- Genotype difference between male age group among healthy
- 30 individuals is significant. For the male population allele significance is

p=0.0009 and genotype significance is p=0.003. The age-related allele and genotype frequency of this marker in both genders and the entire population is shown in Figure 21. The decrease of the homozygous CC genotype in the older male population is highly significant.

- 5        The polymorphism is localized in the non-translated 3'-region of the gene encoding the human methionine sulfoxide reductase (h-msrA). The exact localization is 451 base pairs downstream the stop codon (TAA). It is very likely that this SNP is in linkage disequilibrium (LD) with another polymorphism more upstream in the coding or promoter region; thus, it is
- 10      not directly cause morbidity. The enzyme methionine sulfoxide reductase has been proposed to exhibit multiple biological functions. It may serve to repair oxidative protein damage but also play an important role in the regulation of proteins by activation or inactivation of their biological functions (Moskovitz et al. (1990) PNAS 95:14071-14075). It has also
- 15      been shown that its activity is significantly reduced in brain tissues of Alzheimer patients (Gabbita et al., (1999) J. Neurochem 73:1660-1666). It is scientifically conceivable that proteins involved in the metabolism of reactive oxygen species are associated to disease.

#### CONCLUSION

- 20        The use of the healthy population provides for the identification of morbidity markers. The identification of proteins involved in the G-protein coupled signaling transduction pathway or in the detoxification of oxidative stress can be considered as convincing results. Further confirmation and validation of other potential polymorphisms already
- 25      identified *in silico* in the gene encoding the human protein kinase A anchoring protein could even provide stronger association to morbidity and demonstrate that this gene product is a suitable pharmaceutical or diagnostic target.

**EXAMPLE 4****MALDI-TOF Mass Spectrometry Analysis**

5 All of the products of the enzyme assays listed below were analyzed by MALDI-TOF mass spectrometry. A diluted matrix solution (0.15 $\mu$ L) containing of 10:1 3-hydroxypicolinic acid:ammonium citrate in 1:1 water:acetonitrile diluted 2.5-fold with water was pipetted onto a SpectroChip (Sequenom, Inc.) and was allowed to crystallize. Then, 0.15 $\mu$ L of sample was added. A linear PerSeptive Voyager DE mass spectrometer or Bruker Biflex MALDI-TOF mass spectrometer, operating in  
10 positive ion mode, was used for the measurements. The sample plates were kept at 18.2 kV for 400 nm after each UV laser shot (approximate 250 laser shots total), and then the target voltage was raised to 20 kV. The original spectra were digitized at 500 MHz.

**EXAMPLE 5****15 Sample Conditioning**

Where indicated in the examples below, the products of the enzymatic digestions were purified with ZipTips (Millipore, Bedford, MA). The ZipTips were pre-wetted with 10  $\mu$ L 50% acetonitrile and equilibrated 4 times with 10  $\mu$ L 0.1 M TEAAc. The oligonucleotide fragments were  
20 bound to the C18 in the ZipTip material by continuous aspiration and dispensation of each sample into the ZipTip. Each digested oligonucleotide was conditioned by washing with 10  $\mu$ L 0.1 M TEAAc, followed by 4 washing steps with 10  $\mu$ L H<sub>2</sub>O. DNA fragments were eluted from the ZipTip with 7  $\mu$ L 50% acetonitrile.

25 Any method for condition the samples may be employed. Methods for conditioning, which generally is used to increase peak resolution, are well known (see, *e.g.*, International PCT application No. WO 98/20019).

## EXAMPLE 6

### DNA Glycosylase-Mediated Sequence Analysis

DNA Glycosylases modifies DNA at each position that a specific nucleobase resides in the DNA, thereby producing abasic sites. In a subsequent reaction with another enzyme, a chemical, or heat, the phosphate backbone at each abasic site can be cleaved.

The glycosylase utilized in the following procedures was uracil-DNA glycosylase (UDG). Uracil bases were incorporated into DNA fragments in each position that a thymine base would normally occupy by amplifying a DNA target sequence in the presence of uracil. Each uracil substituted DNA amplicon was incubated with UDG, which cleaved each uracil base in the amplicon, and was then subjected to conditions that effected backbone cleavage at each abasic site, which produced DNA fragments. DNA fragments were subjected to MALDI-TOF mass spectrometry analysis. Genetic variability in the target DNA was then assessed by analyzing mass spectra.

Glycosylases specific for nucleotide analogs or modified nucleotides, as described herein, can be substituted for UDG in the following procedures. The glycosylase methods described hereafter, in conjunction with phosphate backbone cleavage and MALDI, can be used to analyze DNA fragments for the purposes of SNP scanning, bacteria typing, methylation analysis, microsatellite analysis, genotyping, and nucleotide sequencing and re-sequencing.

#### A. Genotyping

A glycosylase procedure was used to genotype the DNA sequence encoding UCP-2 (Uncoupling Protein 2). The sequence for UCP-2 is deposited in GenBank under accession number AF096289. The sequence variation genotyped in the following procedure was a cytosine (C-allele) to thymine (T-allele) variation at nucleotide position 4790, which results in a alanine to valine mutation at position 55 in the UCP-2 polypeptide.

- DNA was amplified using a PCR procedure with a 50  $\mu$ L reaction volume containing of 5 pmol biotinylated primer having the sequence 5'-TGCTTATCCCTGTAGCTACCCTGTCTTGGCCTTGCAGATCCAA-3' (SEQ ID NO: 91), 15 pmol non-biotinylated primer having the sequence 5'-AGCGGATAACAATTTACACAGGCCATCACACCGCGTACTG-3' (SEQ ID NO: 92), 200  $\mu$ M dATP, 200  $\mu$ M dCTP, 200  $\mu$ M dGTP, 600  $\mu$ M dUTP (to fully replace dTTP), 1.5 mM to 3 mM  $MgCl_2$ , 1 U of HotStarTaq polymerase, and 25 ng of CEPH DNA. Amplification was effected with 45 cycles at an annealing temperature of 56°C.
- 10 The amplification product was then immobilized onto a solid support by incubating 50  $\mu$ L of the amplification reaction with 5  $\mu$ L of prewashed Dynabeads for 20 minutes at room temperature. The supernatant was removed, and the beads were incubated with 50  $\mu$ L of 0.1 M NaOH for 5 minutes at room temperature to denature the double-
- 15 stranded PCR product in such a fashion that single-stranded DNA was linked to the beads. The beads were then neutralized by three washes with 50  $\mu$ L 10 mM TrisHCl (pH 8). The beads were resuspended in 10  $\mu$ L of a 60mM TrisHCl/1mM EDTA (pH 7.9) solution, and 1 U uracil DNA glycosylase was added to the solution for 45 minutes at 37°C to remove
- 20 uracil nucleotides present in the single-stranded DNA linked to the beads. The beads were then washed two times with 25  $\mu$ L of 10 mM TrisHCl (pH 8) and once with 10  $\mu$ L of water. The biotinylated strands were then eluted from the beads with 12  $\mu$ L of 2 M  $NH_4OH$  at 60°C for 10 minutes. The backbone of the DNA was cleaved by incubating the samples for 10
- 25 min at 95°C (with a closed lid), and ammonia was evaporated from the samples by incubating the samples for 11 min at 80°C.

The cleavage fragments were then analyzed by MALDI-TOF mass spectrometry as described in Example 4. The T-allele generated a unique fragment of 3254 Daltons. The C-allele generated a unique fragment of 30 4788 Daltons. These fragments were distinguishable in mass spectra.

Thus, the above-identified procedure was successfully utilized to genotype individuals heterozygous for the C-allele and T-allele in UCP-2.

#### **B. Glycosylase Analysis Utilizing Pooled DNA Samples**

The glycosylase assay was conducted using pooled samples to  
5 detect genetic variability at the UCP-2 locus. DNA of known genotype was pooled from eleven individuals and was diluted to a fixed concentration of 5 ng/ $\mu$ L. The procedure provided in Example 3A was followed using 2 pmol of forward primer having a sequence of 5'-  
10 CCCAGTCACGACGTTGTAAAACGTCTTGGCCTTGAGATCCAAG- 3'  
(SEQ ID NO: 93) and 15 pmol of reverse primer having the sequence 5'-  
AGCGGATAACAATTTACACAGGCCATCACACCGCGGTACTG-3' (SEQ  
ID NO: 94). In addition, 5 pmol of biotinylated primer having the  
sequence 5'-bioCCCAGTCACGACGTTGTAAAACG 3' (SEQ ID NO: 97)  
may be introduced to the PCR reaction after about two cycles. The  
15 fragments were analyzed via MALDI-TOF mass spectroscopy (Example 4).  
As determined in Example 3A, the T-allele, which generated a unique  
fragment of 3254 Daltons, could be distinguished in mass spectra from  
the C-allele, which generated a unique fragment of 4788 Daltons. Allelic  
frequency in the pooled samples was quantified by integrating the area  
20 under each signal corresponding to an allelic fragment. Integration was  
accomplished by hand calculations using equations well known to those  
skilled in the art. In the pool of eleven samples, this procedure suggested  
that 40.9% of the individuals harbored the T allele and 59.09% of the  
individuals harbored the C allele.

#### **25 C. Glycosylase-Mediated Microsatellite Analysis**

A glycosylase procedure was utilized to identify microsatellites of  
the Bradykinin Receptor 2 (BKR-2) sequence. The sequence for BKR-2 is  
deposited in GenBank under accession number X86173. BKR-2 includes  
a SNP in the promoter region, which is a C to T variation, as well as a  
30 SNP in a repeated unit, which is a G to T variation. The procedure

provided in Example 3A was utilized to identify the SNP in the promotor region, the SNP in the microsatellite repeat region, and the number of repeated units in the microsatellite region of BKR-2. Specifically, a forward PCR primer having the sequence 5'-

- 5 CTCCAGCTGGGCAGGAGTGC-3' (SEQ ID NO: 95) and a reverse primer having the sequence 5'-CACTTCAGTCGTCCT-3' (SEQ ID NO: 96) were utilized to amplify BKR-2 DNA in the presence of uracil. The amplicon was fragmented by UDG followed by backbone cleavage. The cleavage fragments were analyzed by MALDI-TOF mass spectrometry as  
10 described in Example 4.

- With regard to the SNP in the BKR-2 promotor region having a C to T variation, the C-allele generated a unique fragment having a mass of 7342.4 Daltons and the T-allele generated a unique fragment having a mass of 7053.2 Daltons. These fragments were distinguishable in mass  
15 spectra. Thus, the above-identified procedure was successfully utilized to genotype individuals heterozygous for the C-allele and T-allele in the promotor region of BKR-2.

- With regard to the SNP in the BKR-2 repeat region having a G to T variation, the T-allele generated a unique fragment having a mass of 1784  
20 Daltons, which was readily detected in a mass spectrum. Hence, the presence of the T-allele was indicative of the G to T sequence variation in the repeat region of BKR-2.

- In addition, the number of repeat regions was distinguished between individuals having two repeat sequences and individuals having  
25 three repeat sequences in BKR-2. The DNA of these individuals did not harbor the G to T sequence variation in the repeat sequence as each repeat sequence contained a G at the SNP locus. The number of repeat regions was determined in individual samples by calculating the area under a signal corresponding to a unique DNA fragment having a mass of  
30 2771.6 Daltons. This signal in spectra generated from individuals having

two repeat regions had an area that was thirty-three percent less than the area under the same signal in spectra generated from individuals having three repeat regions. Thus, the procedures discussed above can be utilized to genotype individuals for the number of repeat sequences present in BKR-2.

#### **D. Bisulfite Treatment Coupled with Glycosylase Digestion**

Bisulfite treatment of genomic DNA can be utilized to analyze positions of methylated cytosine residues within the DNA. Treating nucleic acids with bisulfite deaminates cytosine residues to uracil residues, while methylated cytosine remains unmodified. Thus, by comparing the sequence of a PCR product generated from genomic DNA that is not treated with bisulfite with the sequence of a PCR product generated from genomic DNA that is treated with bisulfite, the degree of methylation in a nucleic acid as well as the positions where cytosine is methylated can be deduced.

Genomic DNA (2  $\mu$ g) was digested by incubation with 1  $\mu$ L of a restriction enzyme at 37°C for 2 hours. An aliquot of 3 M NaOH was added to yield a final concentration of 0.3M NaOH in the digestion solution. The reaction was incubated at 37°C for 15 minutes followed by treatment with 5.35M urea, 4.44M bisulfite, and 10mM hydroquinone, where the final concentration of hydroquinone is 0.5 mM.

The sample that was treated with bisulfite (sample A) was compared to the same digestion sample that had not undergone bisulfite treatment (sample B). After sample A was treated with bisulfite as described above, sample A and sample B were amplified by a standard PCR procedure. The PCR procedure included the step of overlaying each sample with mineral oil and then subjecting the sample to thermocycling (20 cycles of 15 minutes at 55°C followed by 30 seconds at 95°C). The PCR reaction contained four nucleotide bases, C, A, G, and U. The mineral oil was removed from each sample, and the PCR products were

purified with glassmilk. Sodium iodide (3 volumes) and glassmilk (5  $\mu$ L) were added to samples A and B. The samples were then placed on ice for 8 minutes, washed with 420  $\mu$ L cold buffer, centrifuged for 10 seconds, and the supernatant fractions were removed. This process was  
5 repeated twice and then 25  $\mu$ L of water was added. Samples were incubated for 5 minutes at 37 °C, were centrifuged for 20 seconds, and the supernatant fraction was collected, and then this incubation/centrifugation/supernatant fraction collection procedure was repeated. 50  $\mu$ L 0.1 M NaOH was then added to the samples to denature  
10 the DNA. The samples were incubated at room temperature for 5 minutes, washed three times with 50  $\mu$ L of 10 mM TrisHCl (pH 8), and resuspended in 10  $\mu$ L 60mM TrisHCl/1mM EDTA, pH 7.9.

The sequence of PCR products from sample A and sample B were then treated with 2U of UDG (MBI Fermentas) and then subjected to  
15 backbone cleavage, as described herein. The resulting fragments from each of sample A and sample B were analyzed by MALDI-TOF mass spectroscopy as described in Example 4. Sample A gave rise to a greater number of fragments than the number of fragments arising from sample B, indicative that the nucleic acid harbored at least one methylated  
20 cytosine moiety.

## EXAMPLE 7

### Fen-Ligase-Mediated Haplotyping

Haplotyping procedures permit the selection of a fragment from one of an individual's two homologous chromosomes and to genotype linked SNPs  
25 on that fragment. The direct resolution of haplotypes can yield increased information content, improving the diagnosis of any linked disease genes or identifying linkages associated with those diseases. In previous studies, haplotypes were typically reconstructed indirectly through pedigree analysis (in cases where pedigrees were available) through

laborious and unreliable allele-specific PCR or through single-molecule dilution methods well known in the art.

5 A haplotyping procedure was used to determine the presence of two SNPs, referred to as SNP1 and SNP2, located on one strand in a DNA sample. The haplotyping procedure used in this assay utilized Fen-1, a site-specific "flap" endonuclease that cleaves DNA "flaps" created by the overlap of two oligonucleotides hybridized to a target DNA strand. The two overlapping oligonucleotides in this example were short arm and long arm allele-specific adaptors. The target DNA was an amplified nucleic acid that had been denatured and contained SNP1 and SNP2.

10 The short arm adaptor included a unique sequence not found in the target DNA. The 3' distal nucleotide of the short arm adaptor was identical to one of the SNP1 alleles. Moreover, the long arm adaptor included two regions: a 3' region complementary to the short arm and a 5' gene-specific region complementary to the fragment of interest adjacent to the SNP. If there was a match between the adaptor and one of the homologues, the Fen enzyme recognized and cleaved the overlapping flap. The short arm of the adaptor was then ligated to the remainder of the target fragment (minus the SNP site). This ligated fragment was used as the forward primer for a second PCR reaction in which only the ligated homologue was amplified. The second PCR product (PCR2) was then analyzed by mass spectrometry. If there was no match between the adaptors and the target DNA, there was no overlap, no cleavage by Fen-1, and thus no PCR2 product of interest.

25 If there was more than one SNP in the sequence of interest, the second SNP (SNP2) was found by using an adaptor that was specific for SNP2 and hybridizing the adaptor to the PCR2 product containing the first SNP. The Fen-ligase and amplification procedures were repeated for the PCR2 product containing the first SNP. If the amplified product yielded a second SNP, then SNP1 and SNP2 were on the same fragment.

If the SNP is unknown, then four allele-specific adaptors (e.g. C, G, A, and T) can be used to hybridize with the target DNA. The substrates are then treated with the Fen-ligase protocol, including amplification. The PCR2 products may be analyzed by PROBE, as described herein, to  
5 determine which adaptors were hybridized to the DNA target and thus identify the SNPs in the sequence.

A Fen-ligase assay was used to detect two SNPs present in Factor VII. These SNPs are located 814 base pairs apart from each other. SNP1 was located at position 8401 (C to T), and SNP2 was located at 9215 (G to A) (SEQ ID #).  
10

#### A. First Amplification Step

A PCR product (PCR1) was generated for a known heterozygous individual at SNP1, a short distance from the 5' end of the SNP. Specifically, a 10  $\mu$ L PCR reaction was performed by mixing 1.5 mM  
15  $\text{MgCl}_2$ , 200  $\mu$ M of each dNTP, 0.5 U HotStar polymerase, 0.1  $\mu$ M of a forward primer having the sequence 5'-GCG CTC CTG TCG GTG CCA (SEQ ID NO: 56), 0.1  $\mu$ M of a reverse primer having the sequence 5'-GCC TGA CTG GTG GGG CCC (SEQ ID NO: 57), and 1 ng of genomic DNA. The annealing temperature was 58°C, and the amplification process  
20 yielded fragments that were 861 bp in length.

The PCR1 reaction mixture was divided in half and was treated with an exonuclease 1/SAP mixture (0.22  $\mu$ L mixture/5  $\mu$ L PCR1 reaction) which contained 1.0  $\mu$ L SAP and 0.1  $\mu$ L exon1. The exonuclease treatment was done for 30 minutes at 37°C and then 20 minutes at  
25 85°C to denature the DNA.

#### B. Adaptor Oligonucleotides

A solution of allele-specific adaptors (C and T), containing of one long and one short oligonucleotide per adaptor, was prepared. The long arm and short arm oligonucleotides of each adaptor (10  $\mu$ M) were mixed in  
30 a 1:1 ratio and heated for 30 seconds at 95°C. The temperature was

reduced in 2°C increments to 37°C for annealing. The C-adaptor had a short arm sequence of 5'-CAT GCA TGC ACG GTC (SEQ ID NO: 58) and a long arm sequence of 5'-CAG AGA GTA CCC CTC GAC CGT GCA TGC ATG (SEQ ID NO: 59). Hence, the long arm of the adaptor was 30 bp (15 bp gene-specific), and the short arm was 15bp. The T-adaptor had a short arm sequence of 5'-CAT GCA TGC ACG GTT (SEQ ID NO: 60) and a long arm sequence of 5'-GTA CGT ACG TGC CAA CTC CCC ATG AGA GAC (SEQ ID NO: 61). The adaptor could also have a hairpin structure in which the short and long arm are separated by a loop containing of 3 to 10 nucleotides (SEQ ID NO: 118).

### C. FEN-ligase reaction

In two tubes (one tube for each allele-specific adaptor per sample) was placed a solution (Solution A) containing of 3.5 µl 10 mM 16%PEG/50 mM MOPS, 1.2 µl 25 mM MgCl<sub>2</sub>, 1.5 µl 10X Ampligase Buffer, and 2.5 µl PCR1. Each tube containing Solution A was incubated at 95°C for 5 minutes to denature the PCR1 product. A second solution (Solution B) containing of 1.65 µl Ampligase (Thermostable ligase, Epicentre Technologies), 1.65 µl 200ng/µl MFEN (from *Methanococcus jannaschii*), and 3.0 µl of an allele specific adaptor (C or T) was prepared. Thus, different variations of Solution B, each variation containing of different allele-specific adaptors, were made. Solution B was added to Solution A at 95°C and incubated at 55°C for 3 hours. The total reaction volume was 15.0 µl per adaptor-specific reaction. For a bi-allelic system, 2 x 15.0 µl reactions were required.

The Fen-ligase reaction in each tube was then deactivated by adding 8.0 µl 10 mM EDTA. Then, 1.0 µl exoIII/Buffer (70%/30%) solution was added to each sample and incubated 30 minutes at 37°C, 20 minutes at 70°C (to deactivate exoIII), and 5 minutes at 95°C (to denature the sample and dissociate unused adaptor from template). The samples were cooled in an ice slurry and purified on UltraClean PCR

Clean-up (MoBio) spin columns which removed all fragments less than 100 base pairs in length. The fragments were eluted with 50  $\mu$ l H<sub>2</sub>O.

#### **D. Second Amplification Step**

- A second amplification reaction (PCR2) was conducted in each
- 5 sample tube using the short arm adaptor (C or T) sequence as the forward primer (minus the SNP1 site). Only the ligated homologue was amplified. A standard PCR reaction was conducted with a total volume of 10.0  $\mu$ l containing of 1X Buffer (final concentration), 1.5 mM final concentration MgCl<sub>2</sub>, 200  $\mu$ M final concentration dNTPs, 0.5 U HotStar polymerase, 0.1
- 10  $\mu$ M final concentration forward primer 5'-CAT GCA TGC ACG GT (SEQ ID NO: 62), 0.1  $\mu$ M final concentration reverse primer 5'-GCC TGA CTG GTG GGG CCC (SEQ ID NO: 63), and 1.0  $\mu$ l of the purified FEN-ligase reaction solution. The annealing temperature was 58°C. The PCR2 product was analyzed by MALDI TOF mass spectroscopy as described in Example 4.
- 15 The mass spectrum of Fen SNP1 showed a mass of 6084.08 Daltons, representing the C allele.

#### **E. Genotyping Additional SNPs**

- The second SNP (SNP2) can be found by using an adaptor that is specific for SNP2 and hybridizing that adaptor to the PCR2 product
- 20 containing the first SNP. The Fen-ligase and amplification procedures are repeated for the PCR2 product containing the first SNP. If the amplified product yields a second SNP, then SN1 and SN2 are on the same fragment. The mass spectrum of SNP2, representing the T allele, showed a mass of 6359.88 Daltons.
- 25 This assay can also be performed upon pooled DNA to yield haplotype frequencies as described herein. The Fen-ligase assay can be used to analyze multiplexes as described herein.

## EXAMPLE 8

### Nickase-Mediated Sequence Analysis

A DNA nickase, or DNase, was used to recognize and cleave one strand of a DNA duplex. Two nickases used were NY2A nickase and NYS1

- 5 nickase (Megabase) which cleave DNA at the following sites:

NY2A: 5'...R AG...3'

3'...Y↓TC...5' where R = A or G and Y = C or T

NYS1: 5'...↓CC[A/G/T]...3'

3'... GG[T/C/A]...5'.

### 10 A. Nickase Digestion

Tris-HCl (10 mM), KCl (10 mM, pH 8.3), magnesium acetate (25 mM), BSA (1 mg/mL), and 6 U of Cvi NY2A or Cvi NYS1 Nickase (Megabase Research) were added to 25 pmol of double-stranded

- 15 TCG TCG CAC TGG GCA TGT G-3' (SEQ ID NO: 90, Operon, Alameda, CA) synthesized using standard phosphoramidite chemistry. With a total volume of 20  $\mu$ L, the reaction mixture was incubated at 37°C for 5 hours, and the digestion products were purified using ZipTips (Millipore, Bedford, MA) as described in Example 5. The samples were analyzed by MALTY-  
20 TOM mass spectroscopy as described in Example 1. The nickase Cvi NY2A yielded three fragments with masses 4049.76 Daltons, 5473.14 Daltons, and 9540.71 Daltons. The Cvi NYS1 nickase yielded fragments with masses 2063.18 Daltons, 3056.48 Daltons, 6492.81 Daltons, and 7450.14 Daltons.

### 25 B. Nickase Digestion of Pooled Samples

DQA (HLA ClassII-DQ Alpha, expected fragment size = 225bp) was amplified from the genomic DNA of 100 healthy individuals. DQA was amplified using standard PCR chemistry in a reaction having a total volume of 50  $\mu$ L containing of 10 mM Tris-HCl, 10 mM KCl (pH 8.3), 2.5  
30 mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP, 10 pmol of a forward primer having

the sequence 5'-GTG CTG CAG GTG TAA ACT TGT ACC AG-3'(SEQ ID NO: 64), 10 pmol of a reverse primer having the sequence 5'-CAC GGA TCC GGT AGC AGC GGT AGA GTT G-3'(SEQ ID NO: 65), 1 U DNA polymerase (Stoffel fragment, Perkin Elmer), and 200ng human genomic DNA (2ng DNA/individual). The template was denatured at 94°C for 5 minutes. Thermal cycling was continued with a touch-down program that included 45 cycles of 20 seconds at 94°C, 30 seconds at 56°C, 1 minute at 72°C, and a final extension of 3 minutes at 72°C. The crude PCR product was used in the subsequent nickase reaction.

The unpurified PCR product was subjected to nickase digestion. Tris-HCl (10 mM), KCl (10 mM, pH 8.3), magnesium acetate (25mM), BSA (1 mg/mL), and 5 U of Cvi NY2A or Cvi NYS1 Nickase (Megabase Research) were added to 25 pmol of the amplified template with a total reaction volume of 20 $\mu$ L. The mixture was then incubated at 37°C for 5 hours. The digestion products were purified with either ZipTips (Millipore, Bedford, MA) as described in Example 5. The samples were analyzed by MALDI-TOF mass spectroscopy as described in Example 4. This assay can also be used to do multiplexing and standardless genotyping as described herein.

To simplify the nickase mass spectrum, the two complementary strands can be separated after digestion by using a single-stranded undigested PCR product as a capture probe. This probe (preparation shown below in Example 8C) can be hybridized to the nickase fragments in hybridization buffer containing 200 mM sodium citrate and 1% blocking reagent (Boehringer Mannheim). The reaction is heated to 95°C for 5 minutes and cooled to room temperature over 30 minutes by using a thermal cycler (PTC-200 DNA engine, MJ Research, Waltham, MA). The capture probe-nickase fragment is immobilized on 140  $\mu$ g of streptavidin-coated magnetic beads. The beads are subsequently washed three times with 70 mM ammonium citrate. The captured single-stranded nickase

fragments are eluted by heating to 80°C for 5 minutes in 5  $\mu$ L of 50 mM ammonium hydroxide.

### C. Preparation of Capture Probe

The capture probe is prepared by amplifying the human  $\beta$ -globin gene (3' end of intron 1 to 5' end of exon 2) via PCR methods in a total volume of 50  $\mu$ L containing of GeneAmp 1XPCR Buffer II, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 10pmol of each primer (forward primer 5'-ACTGGGCATGTGGAGACAG-3'(SEQ ID NO: 66) and biotinylated reverse primer bio5'-GCACCTTCTTGCCATGAG-3'(SEQ ID: 67), 2 U of AmpliTaq Gold, and 200 ng of human genomic DNA. The template is denatured at 94°C for 8 minutes. Thermal cycling is continued with a touch-down program that included 11 cycles of 20 seconds at 94°C, 30 seconds at 64°C, 1 minute at 72°C; and a final extension of 5 minutes at 72°C. The amplicon is purified using UltraClean™ PCR clean-up kit (MO Bio Laboratories, Solano Beach, CA).

## EXAMPLE 9

### Multiplex Type IIS SNP Assay

A Type IIS assay was used to identify human gene sequences with known SNPs. The Type IIS enzyme used in this assay was Fok I which effected double-stranded cleavage of the target DNA. The assay involved the steps of amplification and Fok I treatment of the amplicon. In the amplification step, the primers were designed so that each PCR product of a designated gene target was less than 100 bases such that a Fok I recognition sequence was incorporated at the 5' and 3' end of the amplicon. Therefore, the fragments that were cleaved by Fok I included a center fragment containing the SNP of interest.

Ten human gene targets with known SNPs were analyzed by this assay. Sequences of the ten gene targets, as well as the primers used to amplify the target regions, are found in Table 5. The ten targets were

lipoprotein lipase, prothrombin, factor V, cholesterol ester transfer protein (CETP), factor VII, factor XIII, HLA-H exon 2, HLA-H exon 4, methylenetetrahydrofolate reductase (MTHR), and P53 exon 4 codon 72.

Amplification of the ten human gene sequences were carried out in a single 50  $\mu$ L volume PCR reaction with 20 ng of human genomic DNA template in 5 PCR reaction tubes. Each reaction vial contained 1X PCR buffer (Qiagen), 200  $\mu$ M dNTPs, 1U Hotstar Taq polymerase (Qiagen), 4 mM  $MgCl_2$ , and 10pmol of each primer. US8, having sequence of 5'TCAGTCACGACGTT3'(SEQ ID NO: 68), and US9, having sequence of 5'CGGATAACAATTTC3'(SEQ ID NO: 69), were used for the forward and reverse primers respectively. Moreover, the primers were designed such that a Fok I recognition site was incorporated at the 5' and 3' ends of the amplicon. Thermal cycling was performed in 0.2 mL tubes or a 96 well plate using a MJ Research Thermal Cycler (calculated temperature) with the following cycling parameters: 94°C for 5 minutes; 45 cycles: 94°C for 20 seconds, 56°C for 20 seconds, 72°C for 60 seconds; and 72°C for 3 minutes.

Following PCR, the sample was treated with 0.2 U Exonuclease I (Amersham Pharmacia) and S Alkaline Phosphatase (Amersham Pharmacia) to remove the unincorporated primers and dNTPs. Typically, 0.2 U of exonuclease I and SAP were added to 5  $\mu$ L of the PCR sample. The sample was then incubated at 37°C for 15 minutes. Exonuclease I and SAP were then inactivated by heating the sample up to 85°C for 15 minutes. Fok I digestion was performed by adding 2 U of Fok I (New England Biolab) to the 5  $\mu$ L PCR sample and incubating at 37°C for 30 minutes. Since the Fok I restriction sites are located on both sides of the amplicon, the 5' and 3' cutoff fragments have higher masses than the center fragment containing the SNP. The sample was then purified by anion exchange and analyzed by MALDI-TOF mass spectrometry as described in Example 4. The masses of the gene fragments from this

multiplexing experiment are listed in Table 6. These gene fragments were resolved in mass spectra thereby allowing multiplex analysis of sequence variability in these genes.

**Table 5**  
**Genes for Multiplex Type IIS Assay**

Gene	Sequence	Seq. ID No.	Primers	Seq. ID No.
Lipoprotein Lipase (Asn291Ser)	cctttgagaa agggctctgc ttgattgta gaaagaaccg ctgcaacaat	98-99	5' caatttcacgctctggatgcaatct	70
	ctggagctatg agatcaca-gttaa agtcaagaacc aasegaagca gcaaaatgta		5' caatttcacacagcgagatgctct tttggctctgact 3'	71
Prothrombin	26731 gaattatttt tgttttcta aaactatggt tcccaataaa agtgactctc	100-101	5' tcagtcacgacgttggatgccaa	72
	26781 agcga-alagcctc aatgtcccca gtgtattcca tggagagctc tctggactca		5' taaaagtactctcagc 3' cggatacaaatctggatgacct agagcattgaagc 3'	73
Factor V (Arg506Gln)	taataggact acttctaact tgaagagca gatccctgga caggcga-alagaa	102-103	5' tcagtcacgacgttggatgccaa	74
	atacaggtat tttgtccttg aagtaacctt tcag		5' gatccctggaacaggc 3' cggatacaaatctggatggaca aatatacctgtattcc 3'	75
Cholesterol ester transfer protein (CETP) (I405V)	1261 ctcaccatgg gcatttgatt gcagagcage tccgagctcga-al tccagagcctt	104-105	5' tcagtcacgacgttggatgcaga	76
	1311 cctgcagttca atgatacccg ctgtgggcatt cctgaaggtc atgtctcgtta		5' gcagctccaggtc 3' cagcagatgaatcattgagtcagg aagctctgg 3'	77
Factor VII (R353Q)	1221 agcaaggact cctgaagggg ggacagtggg ggcccaacatg caaccaccta	106-107	5' tcagtcacgacgttggatgccca	78
	1271 gclg-gtgggcaag tggtaacctga caggcatcgt cagctggggc cagggtcgcg		5' catgcacaccacatac 3' cggatacaaatctggatgcccg tccgtaccacg 3'	79
Factor XIII (V34L)	111 caataactct aatgcagcgg aagatgacct gccacacatg agacttaag	108-109	5' tcagtcacgacgttggatgccca	80
	161 gclg-ttgtgtgcc ccggggcttc aacctgcaag gtatgagcat accccccttc		5' cagtgaaggtctcag 3' gctcataccttgcaggaatgagc 3'	81
HLA-H exon 2 (His63Asp)	361 tgaagccttt gggctacgtg gatgaccagc ttctcgtatt ctatgatlc-glat	110-111	5' tcagtcacgacgttggatgccca	82
	411 gaaagtcacc gtgtgagacc ccgaactcca tgggtttcca gtagaatttc		5' gctgtctgtctc 3' tacatggaggttcagggaatgcaca cggcgactctc 3'	83

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Gene	Sequence	Seq. ID No.	Primers	Seq. ID No.
HLA-H exon 4 (Cys282Tyr)	1021 ggataacctt ggctgtaccc <u>ctggggaa</u> <u>agcagagata tacgtg</u> •ajccag	112-113	5' tcagtcacgacgttggatggggg <u>agagcagagataacgt</u> 3'	84
	1071 <u>gtggagcacc caggcctgga tcagccctc</u> attgtatct gggagccctc		5' gggggcctgacccagatgggt <u>gctccac</u> 3'	85
Methylenetetrahydrofolatereductase (MTHFR) (Ala222Val)	761 <u>tgaagcactt gaagga gaag gtgtctgagg</u> <u>gaglc</u> • <u>tlcgattt catcatcag</u>	114-115	5' tcagtcacgacgttggatggggg <u>agagcagagataacgt</u> 3'	86
	811 <u>cagctttctt ttgagcctga cacattcttc</u>		5' gggggcctgacccagatgggt <u>gctccac</u> 3'	87
P53 Exon4 Codon 72 (Arg72Pro)	12101 <u>tccagatgaa gctcccagaa</u> <u>tgcagagggc tgcctccclg</u> •cic gtggcccctg	116-117	5' gatgaagctcccagatgccag <u>aggc</u> 3'	88
	12151 <u>caccagcagc tcttacacgg</u> <u>acggcccctg</u>		5' gccgcggatgaggatgctctg <u>atgc</u> 3'	89

Table 6  
The mass of Center Fragments for Ten Different SNP Typing by IIS Assay

Gene	LPL( <sup>Asn291Ser</sup> )		Prothrombin		FV( <sup>Arg506Gln</sup> )		CETP( <sup>405V</sup> )		FVII( <sup>R353D</sup> )		FXIII( <sup>V34</sup> )	
Genotype	A	G	G	A	G	A	G	A	G	A	G	T
+ strand mass (Da)	6213	6229	5845	5829	5677	5661	3388	3372	6128	6112	5058	5033
- strand mass (Da)	6129	6114	5949	5964	5472	5487	3437	3452	6174	6189	4916	4940

Gene	H1ah2		H1ah4		MTHFR( <sup>Ala222Val</sup> )		P53exon4( <sup>Arg72Pro</sup> )	
Genotype	C	G	G	A	C	T	G	C
+ strand mass (Da)	5889	5929	4392	4376	4400	4415	4586	4546
- strand mass (Da)	5836	5796	4319	4334	4368	4352	4724	4764

**EXAMPLE 10****Exemplary use of parental medical history parameter for stratification of healthy database**

A healthy database can be used to associate a disease state with a specific allele (SNP) that has been found to show a strong association between age and the allele, in particular the homozygous genotype. The method involves using the same healthy database used to identify the age dependent association, however stratification is by information given by the donors about common disorders from which their parents suffered (the donor's familial history of disease). There are three possible answers a donor could give about the health status of their parents: neither were affected, one was affected or both were affected. Only donors above a certain minimum age, depending on the disease, are utilized, as the donors parents must be old enough to to have exhibited clinical disease phenotypes. The genotype frequency in each of these groups is determined and compared with each other. If there is an association of the marker in the donor to a disease the frequency of the heterozygous genotype will be increased. The frequency of the homozygous genotype should not increase, as it should be significantly underrepresented in the healthy population.

**EXAMPLE 11****Method and Device for Identifying a Biological Sample****Description**

- In accordance with the present invention, a method and device for
- 5 identifying a biological sample is provided. Referring now to FIG. 24, an apparatus 10 for identifying a biological sample is disclosed. The apparatus 10 for identifying a biological sample generally comprises a mass spectrometer 15 communicating with a computing device 20. In a preferred embodiment, the mass spectrometer may be a MALDI-TOF mass spectrometer manufactured by
- 10 Bruker-Franzen Analytik GmbH; however, it will be appreciated that other mass spectrometers can be substituted. The computing device 20 is preferably a general purpose computing device. However, it will be appreciated that the computing device could be alternatively configured, for example, it may be

integrated with the mass spectrometer or could be part of a computer in a larger network system.

The apparatus 10 for identifying a biological sample may operate as an automated identification system having a robot 25 with a robotic arm 27 configured to deliver a sample plate 29 into a receiving area 31 of the mass spectrometer 15. In such a manner, the sample to be identified may be placed on the plate 29 and automatically received into the mass spectrometer 15. The biological sample is then processed in the mass spectrometer to generate data indicative of the mass of DNA fragments in the biological sample. This data may be sent directly to computing device 20, or may have some preprocessing or filtering performed within the mass spectrometer. In a preferred embodiment, the mass spectrometer 15 transmits unprocessed and unfiltered mass spectrometry data to the computing device 20. However, it will be appreciated that the analysis in the computing device may be adjusted to accommodate preprocessing or filtering performed within the mass spectrometer.

Referring now to FIG. 25, a general method 35 for identifying a biological sample is shown. In method 35, data is received into a computing device from a test instrument in block 40. Preferably the data is received in a raw, unprocessed and unfiltered form, but alternatively may have some form of filtering or processing applied. The test instrument of a preferred embodiment is a mass spectrometer as described above. However, it will be appreciated that other test instruments could be substituted for the mass spectrometer.

The data generated by the test instrument, and in particular the mass spectrometer, includes information indicative of the identification of the biological sample. More specifically, the data is indicative of the DNA composition of the biological sample. Typically, mass spectrometry data gathered from DNA samples obtained from DNA amplification techniques are noisier than, for example, those from typical protein samples. This is due in part because protein samples are more readily prepared in more abundance, and protein samples are more easily ionizable as compared to DNA samples. Accordingly, conventional mass spectrometer data analysis techniques are generally ineffective for DNA analysis of a biological sample. To improve the

analysis capability so that DNA composition data can be more readily discerned, a preferred embodiment uses wavelet technology for analyzing the DNA mass spectrometry data. Wavelets are an analytical tool for signal processing, numerical analysis, and mathematical modeling. Wavelet technology provides a basic expansion function which is applied to a data set. Using wavelet decomposition, the data set can be simultaneously analyzed in the time and frequency domains. Wavelet transformation is the technique of choice in the analysis of data that exhibit complicated time (mass) and frequency domain information, such as MALDI-TOF DNA data. Wavelet transforms as described herein have superior denoising properties as compared to conventional Fourier analysis techniques. Wavelet transformation has proven to be particularly effective in interpreting the inherently noisy MALDI-TOF spectra of DNA samples. In using wavelets, a "small wave" or "scaling function" is used to transform a data set into stages, with each stage representing a frequency component in the data set. Using wavelet transformation, mass spectrometry data can be processed, filtered, and analyzed with sufficient discrimination to be useful for identification of the DNA composition for a biological sample.

Referring again to FIG. 25, the data received in block 40 is denoised in block 45. The denoised data then has a baseline correction applied in block 50. A baseline correction is generally necessary as data coming from the test instrument, in particular a mass spectrometer instrument, has data arranged in a generally exponentially decaying manner. This generally exponential decaying arrangement is not due to the composition of the biological sample, but is a result of the physical properties and characteristics of the test instrument, and other chemicals involved in DNA sample preparation. Accordingly, baseline correction substantially corrects the data to remove a component of the data attributable to the test system, and sample preparation characteristics.

After denoising in block 45 and the baseline correction in block 50, a signal remains which is generally indicative of the composition of the biological sample. However, due to the extraordinary discrimination required for analyzing the DNA composition of the biological sample, the composition is not readily apparent from the denoised and corrected signal. For example, although the

signal may include peak areas, it is not yet clear whether these "putative" peaks actually represent a DNA composition, or whether the putative peaks are result of a systemic or chemical aberration. Further, any call of the composition of the biological sample would have a probability of error which would be unacceptable for clinical or therapeutic purposes. In such critical situations, there needs to be a high degree of certainty that any call or identification of the sample is accurate. Therefore, additional data processing and interpretation is necessary before the sample can be accurately and confidently identified.

Since the quantity of data resulting from each mass spectrometry test is typically thousands of data points, and an automated system may be set to perform hundreds or even thousands of tests per hour, the quantity of mass spectrometry data generated is enormous. To facilitate efficient transmission and storage of the mass spectrometry data, block 55 shows that the denoised and baseline corrected data is compressed.

In a preferred embodiment, the biological sample is selected and processed to have only a limited range of possible compositions. Accordingly, it is therefore known where peaks indicating composition should be located, if present. Taking advantage of knowing the location of these expected peaks, in block 60 the method 35 matches putative peaks in the processed signal to the location of the expected peaks. In such a manner, the probability of each putative peak in the data being an actual peak indicative of the composition of the biological sample can be determined. Once the probability of each peak is determined in block 60, then in block 65 the method 35 statistically determines the composition of the biological sample, and determines if confidence is high enough to calling a genotype.

Referring again to block 40, data is received from the test instrument, which is preferably a mass spectrometer. In a specific illustration, FIG. 26 shows an example of data from a mass spectrometer. The mass spectrometer data 70 generally comprises data points distributed along an x-axis 71 and a y-axis 72. The x-axis 71 represents the mass of particles detected, while the y-axis 72 represents a numerical concentration of the particles. As can be seen in FIG. 26, the mass spectrometry data 70 is generally exponentially decaying with

data at the left end of the x-axis 73 generally decaying in an exponential manner toward data at the heavier end 74 of the x-axis 71. However, the general exponential presentation of the data is not indicative of the composition of the biological sample, but is more reflective of systematic error and characteristics.

- 5 Further, as described above and illustrated in FIG. 26, considerable noise exists in the mass spectrometry DNA data 70.

Referring again to block 45, where the raw data received in block 40 is denoised, the denoising process will be described in more detail. As illustrated in FIG. 25, the denoising process generally entails 1) performing a wavelet  
10 transformation on the raw data to decompose the raw data into wavelet stage coefficients; 2) generating a noise profile from the highest stage of wavelet coefficients; and 3) applying a scaled noise profile to other stages in the wavelet transformation. Each step of the denoising process is further described below.

Referring now to FIG. 27, the wavelet transformation of the raw mass  
15 spectrometry data is generally diagramed. Using wavelet transformation techniques, the mass spectrometry data 70 is sequentially transformed into stages. In each stage the data is represented in a high stage and a low stage, with the low stage acting as the input to the next sequential stage. For example, the mass spectrometry data 70 is transformed into stage 0 high data 82 and stage 0 low data 83. The stage 0 low data 83 is then used as an input  
20 to the next level transformation to generate stage 1 high data 84 and stage 1 low data 85. In a similar manner, the stage 1 low data 85 is used as an input to be transformed into stage 2 high data 86 and stage 2 low data 87. The transformation is continued until no more useful information can be derived by  
25 further wavelet transformation. For example, in the preferred embodiment a 24-point wavelet is used. More particularly a wavelet commonly referred to as the Daubechies 24 is used to decompose the raw data. However, it will be appreciated that other wavelets can be used for the wavelet transformation. Since each stage in a wavelet transformation has one-half the data points of the  
30 previous stage, the wavelet transformation can be continued until the stage n low data 89 has around 50 points. Accordingly, the stage n high 88 would contain about 100 data points. Since the preferred wavelet is 24 points long,

little data or information can be derived by continuing the wavelet transformation on a data set of around 50 points.

FIG. 28 shows an example of stage 0 high data 95. Since stage 0 high data 95 is generally indicative of the highest frequencies in the mass spectrometry data, stage 0 high data 95 will closely relate to the quantity of high frequency noise in the mass spectrometry data. In FIG. 29, an exponential fitting formula has been applied to the stage 0 high data 95 to generate a stage 0 noise profile 97. In particular, the exponential fitting formula is in the format  $A_0 + A_1 \text{EXP}(-A_2 m)$ . It will be appreciated that other exponential fitting formulas or other types of curve fits may be used.

Referring now to FIG. 30, noise profiles for the other high stages are determined. Since the later data points in each stage will likely be representative of the level of noise in each stage, only the later data points in each stage are used to generate a standard deviation figure that is representative of the noise content in that particular stage. More particularly, in generating the noise profile for each remaining stage, only the last five percent of the data points in each stage are analyzed to determine a standard deviation number. It will be appreciated that other numbers of points, or alternative methods could be used to generate such a standard deviation figure.

The standard deviation number for each stage is used with the stage 0 noise profile (the exponential curve) 97 to generate a scaled noise profile for each stage. For example, FIG. 30 shows that stage 1 high data 98 has stage 1 high data 103 with the last five percent of the data points represented by area 99. The points in area 99 are evaluated to determine a standard deviation number indicative of the noise content in stage 1 high data 103. The standard deviation number is then used with the stage 0 noise profile 97 to generate a stage 1 noise profile.

In a similar manner, stage 2 high 100 has stage 2 high data 104 with the last five percent of points represented by area 101. The data points in area 101 are then used to calculate a standard deviation number which is then used to scale the stage 0 noise profile 97 to generate a noise profile for stage 2 data. This same process is continued for each of the stage high data as shown by the

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data 70 has been denoised and is ready for further processing. A wavelet transformation of the denoised signal results in the sparse data set 120 as shown in FIG. 31.

Referring again to FIG. 25, the mass spectrometry data received in block 40 has been denoised in block 45 and is now passed to block 50 for baseline correction. Before performing baseline correction, the artifacts introduced by the wavelet transformation procedure are preferably removed. Wavelet transformation results vary slightly depending upon which point of the wavelet is used as a starting point. For example, the preferred embodiment uses the 24-point Daubechies-24 wavelet. By starting the transformation at the 0 point of the wavelet, a slightly different result will be obtained than if starting at points 1 or 2 of the wavelet. Therefore, the denoised data is transformed using every available possible starting point, with the results averaged to determine a final denoised and shifted signal. For example, FIG. 33 shows that the wavelet coefficient is applied 24 different times and then the results averaged to generate the final data set. It will be appreciated that other techniques may be used to accommodate the slight error introduced due to wavelet shifting.

The formula 125 is generally indicated in FIG. 33. Once the signal has been denoised and shifted, a denoised and shifted signal 130 is generated as shown in FIG. 58. FIG. 34 shows an example of the wavelet coefficient 135 data set from the denoised and shifted signal 130.

FIG. 36 shows that putative peak areas 145, 147, and 149 are located in the denoised and shifted signal 150. The putative peak areas are systematically identified by taking a moving average along the signal 150 and identifying sections of the signal 150 which exceed a threshold related to the moving average. It will be appreciated that other methods can be used to identify putative peak areas in the signal 150.

Putative peak areas 145, 147 and 149 are removed from the signal 150 to create a peak-free signal 155 as shown in FIG. 37. The peak-free signal 155 is further analyzed to identify remaining minimum values 157, and the remaining minimum values 157 are connected to generate the peak-free signal 155.

FIG. 38 shows a process of using the peak-free signal 155 to generate a baseline 170 as shown in FIG. 39. As shown in block 162, a wavelet transformation is performed on the peak-free signal 155. All the stages from the wavelet transformation are eliminated in block 164 except for the n low stage.

- 5 The n low stage will generally indicate the lowest frequency component of the peak-free signal 155 and therefore will generally indicate the system exponential characteristics. Block 166 shows that a signal is reconstructed from the n low coefficients and the baseline signal 170 is generated in block 168.

- FIG. 39 shows a denoised and shifted data signal 172 positioned adjacent  
10 a correction baseline 170. The baseline correction 170 is subtracted from the denoised and shifted signal 172 to generate a signal 175 having a baseline correction applied as shown in FIG. 40. Although such a denoised, shifted, and corrected signal is sufficient for most identification purposes, the putative peaks in signal 175 are not identifiable with sufficient accuracy or confidence to call  
15 the DNA composition of a biological sample.

- Referring again to FIG. 25, the data from the baseline correction 50 is now compressed in block 55, the compression technique used in a preferred embodiment is detailed in FIG. 41. In FIG. 41 the data in the baseline corrected data is presented in an array format 182 with x-axis points 183 having an  
20 associated data value 184. The x-axis is indexed by the non-zero wavelet coefficients, and the associated value is the value of the wavelet coefficient. In the illustrated data example in table 182, the maximum value 184 is indicated to be 1000. Although a particularly advantageous compression technique for mass spectrometry data is shown, it will be appreciated that other compression  
25 techniques can be used. Although not preferred, the data may also be stored without compression.

- In compressing the data according to a preferred embodiment, an intermediate format 186 is generated. The intermediate format 186 generally comprises a real number having a whole number portion 188 and a decimal  
30 portion 190. The whole number portion is the x-axis point 183 while the decimal portion is the value data 184 divided by the maximum data value. For

example, in the data 182 a data value "25" is indicated at x-axis point "100". The intermediate value for this data point would be "100.025".

From the intermediate compressed data 186 the final compressed data 195 is generated. The first point of the intermediate data file becomes the starting point for the compressed data. Thereafter each data point in the compressed data 195 is calculated as follows: the whole number portion (left of the decimal) is replaced by the difference between the current and the last whole number. The remainder (right of the decimal) remains intact. For example, the starting point of the compressed data 195 is shown to be the same as the intermediate data point which is "100.025". The comparison between the first intermediate data point "100.025" and the second intermediate data point "150.220" is "50.220". Therefore, "50.220" becomes the second point of the compressed data 195. In a similar manner, the second intermediate point is "150.220" and the third intermediate data point is "500.0001". Therefore, the third compressed data becomes "350.000". The calculation for determining compressed data points is continued until the entire array of data points is converted to a single array of real numbers.

FIG. 42 generally describes the method of compressing mass spectrometry data, showing that the data file in block 201 is presented as an array of coefficients in block 202. The data starting point and maximum is determined as shown in block 203, and the intermediate real numbers are calculated in block 204 as described above. With the intermediate data points generated, the compressed data is generated in block 205. The described compression method is highly advantageous and efficient for compressing data sets such as a processed data set from a mass spectrometry instrument. The method is particularly useful for data, such as mass spectrometry data, that uses large numbers and has been processed to have occasional lengthy gaps in x-axis data. Accordingly, an x-y data array for processed mass spectrometry data may be stored with an effective compression rate of 10x or more. Although the compression technique is applied to mass spectrometry data, it will be appreciated that the method may also advantageously be applied to other data sets.

Referring again to FIG. 25, peak heights are now determined in block 60. The first step in determining peak height is illustrated in FIG. 43 where the signal 210 is shifted left or right to correspond with the position of expected peaks. As the set of possible compositions in the biological sample is known before the mass spectrometry data is generated, the possible positioning of expected peaks is already known. These possible peaks are referred to as expected peaks, such as expected peaks 212, 214, and 216. Due to calibration or other errors in the test instrument data, the entire signal may be shifted left or right from its actual position, therefore, putative peaks located in the signal, such as putative peaks 218, 222, and 224 may be compared to the expected peaks 212, 214, and 216, respectively. The entire signal is then shifted such that the putative peaks align more closely with the expected peaks.

Once the putative peaks have been shifted to match expected peaks, the strongest putative peak is identified in FIG. 44. In a preferred embodiment, the strongest peak is calculated as a combination of analyzing the overall peak height and area beneath the peak. For example, a moderately high but wide peak would be stronger than a very high peak that is extremely narrow. With the strongest putative peak identified, such as putative peak 225, a Gaussian 228 curve is fit to the peak 225. Once the Gaussian is fit, the width (W) of the Gaussian is determined and will be used as the peak width for future calculations.

As generally addressed above, the denoised, shifted, and baseline-corrected signal is not sufficiently processed for confidently calling the DNA composition of the biological sample. For example, although the baseline has generally been removed, there are still residual baseline effects present. These residual baseline effects are therefore removed to increase the accuracy and confidence in making identifications.

To remove the residual baseline effects, FIG. 45 shows that the putative peaks 218, 222, and 224 are removed from the baseline corrected signal. The peaks are removed by identifying a center line 230, 232, and 234 of the putative peaks 218, 222, and 224, respectively and removing an area to the left and to the right of the identified center line. For each putative peak, an area

equal to twice the width (W) of the Gaussian is removed from the left of the center line, while an area equivalent to 50 daltons is removed from the right of the center line. It has been found that the area representing 50 daltons is adequate to sufficiently remove the effect of salt adducts which may be associated with an actual peak. Such adducts appear to the right of an actual peak and are a natural effect from the chemistry involved in acquiring a mass spectrum. Although a 50 Dalton buffer has been selected, it will be appreciated that other ranges or methods can be used to reduce or eliminate adduct effects.

The peaks are removed and remaining minima 247 located as shown in FIG. 46 with the minima 247 connected to create signal 245. A quartic polynomial is applied to signal 245 to generate a residual baseline 250 as shown in FIG. 47. The residual baseline 250 is subtracted from the signal 225 to generate the final signal 255 as indicated in FIG. 48. Although the residual baseline is the result of a quartic fit to signal 245, it will be appreciated that other techniques can be used to smooth or fit the residual baseline.

To determine peak height, as shown in FIG. 49, a Gaussian such as Gaussian 266, 268, and 270 is fit to each of the peaks, such as peaks 260, 262, and 264, respectively. Accordingly, the height of the Gaussian is determined as height 272, 274, and 276. Once the height of each Gaussian peak is determined, then the method of identifying a biological compound can move into the genotyping phase 65 as shown in FIG. 25.

An indication of the confidence that each putative peak is an actual peak can be discerned by calculating a signal-to-noise ratio for each putative peak. Accordingly, putative peaks with a strong signal-to-noise ratio are generally more likely to be an actual peak than a putative peak with a lower signal-to-noise ratio. As described above and shown in FIG. 50, the height of each peak, such as height 272, 274, and 276, is determined for each peak, with the height being an indicator of signal strength for each peak. The noise profile, such as noise profile 97, is extrapolated into noise profile 280 across the identified peaks. At the center line of each of the peaks, a noise value is determined, such as noise value 282, 283, and 284. With a signal values and a noise values generated, signal-to-noise ratios can be calculated for each peak. For example, the signal-

to-noise ratio for the first peak in FIG. 50 would be calculated as signal value 272 divided by noise value 282, and in a similar manner the signal-to-noise ratio of the middle peak in FIG. 50 would be determined as signal 274 divided by noise value 283.

5 Although the signal-to-noise ratio is generally a useful indicator of the presence of an actual peak, further processing has been found to increase the confidence by which a sample can be identified. For example, the signal-to-noise ratio for each peak in the preferred embodiment is preferably adjusted by the goodness of fit between a Gaussian and each putative peak. It is a  
10 characteristic of a mass spectrometer that sample material is detected in a manner that generally complies with a normal distribution. Accordingly, greater confidence will be associated with a putative signal having a Gaussian shape than a signal that has a less normal distribution. The error resulting from having a non-Gaussian shape can be referred to as a "residual error".

15 Referring to FIG. 51, a residual error is calculated by taking a root mean square calculation between the Gaussian 293 and the putative peak 290 in the data signal. The calculation is performed on data within one width on either side of a center line of the Gaussian. The residual error is calculated as:

20 where G is the Gaussian signal value, R is the putative peak value, and N is the number of points from  $-W$  to  $+W$ . The calculated residual error is used to generate an adjusted signal-to-noise ratio, as described below.

An adjusted signal noise ratio is calculated for each putative peak using the formula  $(S/N) * \text{EXP}^{(-.1 * R)}$ , where S/N is the signal-to-noise ratio, and R is the residual error determined above. Although the preferred embodiment  
25 calculates an adjusted signal-to-noise ratio using a residual error for each peak, it will be appreciated that other techniques can be used to account for the goodness of fit between the Gaussian and the actual signal.

Referring now to FIG. 52, a probability is determined that a putative peak is an actual peak. In making the determination of peak probability, a probability  
30 profile 300 is generated where the adjusted signal-to-noise ratio is the x-axis and the probability is the y-axis. Probability is necessarily in the range between a 0% probability and a 100% probability, which is indicated as 1. Generally, the

higher the adjusted signal-to-noise ratio, the greater the confidence that a putative peak is an actual peak.

- At some target value for the adjusted signal-to-noise, it has been found that the probability is 100% that the putative peak is an actual peak and can confidently be used to identify the DNA composition of a biological sample. However, the target value of adjusted signal-to-noise ratio where the probability is assumed to be 100% is a variable parameter which is to be set according to application specific criteria. For example, the target signal-to-noise ratio will be adjusted depending upon trial experience, sample characteristics, and the acceptable error tolerance in the overall system. More specifically, for situations requiring a conservative approach where error cannot be tolerated, the target adjusted signal-to-noise ratio can be set to, for example, 10 and higher. Accordingly, 100% probability will not be assigned to a peak unless the adjusted signal-to-noise ratio is 10 or over.
- In other situations, a more aggressive approach may be taken as sample data is more pronounced or the risk of error may be reduced. In such a situation, the system may be set to assume a 100% probability with a 5 or greater target signal-to-noise ratio. Of course, an intermediate signal-to-noise ratio target figure can be selected, such as 7, when a moderate risk of error can be assumed. Once the target adjusted signal-to-noise ratio is set for the method, then for any adjusted signal-to-noise ratio a probability can be determined that a putative peak is an actual peak.

- Due to the chemistry involved in performing an identification test, especially a mass spectrometry test of a sample prepared by DNA amplifications, the allelic ratio between the signal strength of the highest peak and the signal strength of the second (or third and so on) highest peak should fall within an expected ratio. If the allelic ratio falls outside of normal guidelines, the preferred embodiment imposes an allelic ratio penalty to the probability. For example, FIG. 53 shows an allelic ratio 315 which has an x-axis 317 that is the ratio between the signal strength of the second highest peak divided by signal strength of the highest peak. The y-axis 319 assigns a penalty between 0 and 1 depending on the determined allelic ratio. In the preferred embodiment, it is

5 315 is used to determine a penalty 319, which is multiplied by the peak probability determined in FIG. 52 to determine a final peak probability. Although the preferred embodiment incorporates an allelic ratio penalty to account for a possible chemistry error, it will be appreciated that other techniques may be used. Similar treatment will be applied to the other peaks.

With the peak probability of each peak determined, the statistical probability for various composition components may be determined. As an example, in order to determine the probability of each of three possible combinations of two peaks, -- peak G, peak C and combinations GG, CC and GC. FIG. 54 shows an example where a most probable peak 325 is determined to have a final peak probability of 90%. Peak 325 is positioned such that it represents a G component in the biological sample. Accordingly, it can be maintained that there is a 90% probability that G exists in the biological sample. Also in the example shown in FIG. 54, the second highest probability is peak 330 which has a peak probability of 20%. Peak 330 is at a position associated with a C composition. Accordingly, it can be maintained that there is a 20% probability that C exists in the biological sample.

With the probability of G existing (90%) and the probability of C existing (20%) as a starting point, the probability of combinations of G and C existing can be calculated. For example, FIG. 54 indicates that the probability of GG 25 existing 329 is calculated as 72%. This is calculated as the probability of GG is equal to the probability of G existing (90%) multiplied by the probability of C not existing (100% - 20%). So if the probability of G existing is 90% and the probability of C not existing is 80%, the probability of GG is 72%.

In a similar manner, the probability of CC existing is equivalent to the probability of C existing (20%) multiplied by the probability of G not existing (100% - 90%). As shown in FIG. 54, the probability of C existing is 20% while the probability of G not existing is 10%, so therefore the probability of CC is

only 2%. Finally, the probability of GC existing is equal to the probability of G existing (90%) multiplied by the probability of C existing (20%). So if the probability of G existing is 90% and the probability of C existing is 20%, the probability of GC existing is 18%. In summary form, then, the probability of the

5 composition of the biological sample is:

probability of GG: 72%;  
probability of GC: 18%; and  
probability of CC: 2%.

Once the probabilities of each of the possible combinations has been  
10 determined, FIG. 55 is used to decide whether or not sufficient confidence exists to call the genotype. FIG. 55 shows a call chart 335 which has an x-axis 337 which is the ratio of the highest combination probability to the second highest combination probability. The y-axis 339 simply indicates whether the ratio is sufficiently high to justify calling the genotype. The value of the ratio may be  
15 indicated by M 340. The value of M is set depending upon trial data, sample composition, and the ability to accept error. For example, the value M may be set relatively high, such as to a value 4 so that the highest probability must be at least four times greater than the second highest probability before confidence is established to call a genotype. However, if a certain level of error may be  
20 acceptable, the value of M may be set to a more aggressive value, such as to 3, so that the ratio between the highest and second highest probabilities needs to be only a ratio of 3 or higher. Of course, moderate value may be selected for M when a moderate risk can be accepted. Using the example of FIG. 54, where the probability of GG was 72% and the probability of GC was 18%, the ratio  
25 between 72% and 18% is 4.0, therefore, whether M is set to 3, 3.5, or 4, the system would call the genotype as GG. Although the preferred embodiment uses a ratio between the two highest peak probabilities to determine if a genotype confidently can be called, it will be appreciated that other methods may be substituted. It will also be appreciated that the above techniques may  
30 be used for calculating probabilities and choosing genotypes (or more general DNA patterns) containing of combinations of more than two peaks.

Referring now to FIG. 56, a flow chart is shown generally defining the process of statistically calling genotype described above. In FIG. 56 block 402 shows that the height of each peak is determined and that in block 404 a noise profile is extrapolated for each peak. The signal is determined from the height of each peak in block 406 and the noise for each peak is determined using the noise profile in block 408. In block 410, the signal-to-noise ratio is calculated for each peak. To account for a non-Gaussian peak shape, a residual error is determined in block 412 and an adjusted signal-to-noise ratio is calculated in block 414. Block 416 shows that a probability profile is developed, with the probability of each peak existing found in block 418. An allelic penalty may be applied in block 420, with the allelic penalty applied to the adjusted peak probability in block 422. The probability of each combination of components is calculated in block 424 with the ratio between the two highest probabilities being determined in block 426. If the ratio of probabilities exceeds a threshold value then the genotype is called in block 428.

In another embodiment of the invention, the computing device 20 (Fig. 24) supports "standardless" genotyping by identifying data peaks that contain putative SNPs. Standardless genotyping is used, for example, where insufficient information is known about the samples to determine a distribution of expected peak locations, against which an allelic penalty as described above can be reliably calculated. This permits the computing device to be used for identification of peaks that contain putative SNPs from data generated by any assay that fragments a targeted DNA molecule. For such standardless genotyping, peaks that are associated with an area under the data curve that deviates significantly from the typical area of other peaks in the data spectrum are identified and their corresponding mass (location along the x-axis) is determined.

More particularly, peaks that deviate significantly from the average area of other peaks in the data are identified, and the expected allelic ratio between data peaks is defined in terms of the ratio of the area under the data peaks. Theoretically, where each genetic loci has the same molar concentration of

analyte, the area under each corresponding peak should be the same, thus producing a 1.0 ratio of the peak area between any two peaks. In accordance with the invention, peaks having a smaller ratio relative to the other peaks in the data will not be recognized as peaks. More particularly, peaks having an area  
5 ratio smaller than 30% relative to a nominal value for peak area will be assigned an allelic penalty. The mass of the remaining peaks (their location along the x-axis of the data) will be determined based on oligonucleotide standards.

Fig. 57 shows a flow diagram representation of the processing by the computing device 20 (Fig. 24) when performing standardless genotyping. In the  
10 first operation, represented by the flow diagram box numbered 502, the computing device receives data from the mass spectrometer. Next, the height of each putative peak in the data sample is determined, as indicated by the block 504. After the height of each peak in the mass spectrometer data is determined, a de-noise process 505 is performed, beginning with an  
15 extrapolation of the noise profile (block 506), followed by finding the noise of each peak (block 508) and calculating the signal to noise ratio for each data sample (block 510). Each of these operations may be performed in accordance with the description above for denoise operations 45 of Fig. 25. Other suitable denoise operations will occur to those skilled in the art.

20 The next operation is to find the residual error associated with each data point. This is represented by the block 512 in Figure 57. The next step, block 514, involves calculating an adjusted signal to noise ratio for each identified peak. A probability profile is developed next (block 516), followed by a determination of the peak probabilities at block 518. In the preferred  
25 embodiment, the denoise operations of Fig. 57, comprising block 502 to block 518, comprise the corresponding operations described above in conjunction with Fig. 56 for block 402 through block 418, respectively.

The next action for the standardless genotype processing is to determine an allelic penalty for each peak, indicated by the block 524. As noted above,  
30 the standardless genotype processing of Fig. 57 determines an allelic penalty by comparing area under the peaks. Therefore, rather than compare signal strength ratios to determine an allelic penalty, such as described above for Fig. 53, the

standardless processing determines the area under each of the identified peaks and compares the ratio of those areas. Determining the area under each peak may be computed using conventional numerical analysis techniques for calculating the area under a curve for experimental data.

Thus, the allelic penalty is assigned in accordance with Fig. 58, which shows that no penalty is assigned to peaks having a peak area relative to an expected average area value that is greater than 0.30 (30%). The allelic penalty is applied to the peak probability value, which may be determined according to the process such as described in Fig. 52. It should be apparent from Fig. 58 that the allelic penalty imposed for peaks below a ratio of 30% is that such peaks will be removed from further measurement and processing. Other penalty schemes, however, may be imposed in accordance with knowledge about the data being processed, as determined by those skilled in the art.

After the allelic penalty has been determined and applied, the standardless genotype processing compares the location of the remaining putative peaks to oligonucleotide standards to determine corresponding masses in the processing for block 524. For standardless genotype data, the processing of the block 524 is performed to determine mass and genotype, rather than performing the operations corresponding to block 424, 426, and 428 of Fig. 33. Techniques for performing such comparisons and determining mass will be known to those skilled in the art.

In another embodiment, the computing device 20 (Fig. 24) permits the detection and determination of the mass (location along the x-axis of the data) of the sense and antisense strand of fragments generated in the assay. If desired, the computing device may also detect and determine the quantity (area under each peak) of the respective sense and antisense strands, using a similar technique to that described above for standardless genotype processing. The data generated for each type of strand may then be combined to achieve a data redundancy and to thereby increase the confidence level of the determined genotype. This technique obviates primer peaks that are often observed in data from other diagnostic methods, thereby permitting a higher level of multiplexing. In addition, when quantitation is used in pooling experiments, the ratio of the

measured peak areas is more reliably calculated than the peak identifying technique, due to data redundancy.

Fig. 23 is a flow diagram that illustrates the processing implemented by the computing device 20 to perform sense and antisense processing. In the first operation, represented by the flow diagram box numbered 602, the computing device receives data from the mass spectrometer. This data will include data for the sense strand and antisense strand of assay fragments. Next, the height of each putative peak in the data sample is determined, as indicated by the block 604. After the height of each peak in the mass spectrometer data is determined, a de-noise process 605 is performed, beginning with an operation that extrapolates the noise profile (block 606), followed by finding the noise of each peak (block 608) and calculating the signal to noise ratio for each data sample (block 610). Each of these operations may be performed in accordance with the description above for the denoise operations 45 of Fig. 25. Other suitable denoise operations will occur to those skilled in the art. The next operation is to find the residual error associated with each data point. This is represented by the block 612 in Figure 36.

After the residual error for the data of the sense strand and antisense strand has been performed, processing to identify the genotypes will be performed for the sense strand and also for the antisense strand. Therefore, Fig. 23 shows that processing includes sense strand processing (block 630) and antisense strand processing (block 640). Each block 630, 640 includes processing that corresponds to adjusting the signal to noise ratio, developing a probability profile, determining an allelic penalty, adjusting the peak probability by the allelic penalty, calculating genotype probabilities, and testing genotype probability ratios, such as described above in conjunction with blocks 414 through 426 of Fig. 56. The processing of each block 630, 640 may, if desired, include standardless processing operations such as described above in conjunction with Fig. 57. The standardless processing may be included in place of or in addition to the processing operations of Fig. 56.

After the genotype probability processing is completed, the data from the sense strand and antisense strand processing is combined and compared to

expected database values to obtain the benefits of data redundancy as between the sense strand and antisense strand. Those skilled in the art will understand techniques to take advantage of known data redundancies between a sense strand and antisense strand of assay fragments. This processing is represented  
5 by the block 650. After the data from the two strands is combined for processing, the genotype processing is performed (block 660) and the genotype is identified.

Since modifications will be apparent to those of skill in this art, it is  
10 intended that this invention be limited only by the scope of the appended claims.

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**WHAT IS CLAIMED IS:**

1. A subcollection of samples from a target population, comprising:  
a plurality of samples, wherein the samples are selected from the group  
consisting of blood, tissue, body fluid, cell, seed, microbe, pathogen and  
5 reproductive tissue samples; and  
a symbology on the containers containing the samples, wherein the  
symbology is representative of the source and/or history of each sample,  
wherein:  
the target population is a healthy population that has not been selected  
10 for any disease state;  
the collection comprises samples from the healthy population; and  
the subcollection is obtained by sorting the collection according to  
specified parameters.
2. The subcollection of claim 1, wherein the parameters are selected  
15 from the group consisting of ethnicity, age, gender, height, weight, alcohol  
intake, number of pregnancies, number of live births, vegetarians, type of  
physical activity, state of residence and/or length of residence in a particular  
state, educational level, age of parent at death, cause of parent death, former or  
current smoker, length of time as a smoker, frequency of smoking, occurrence  
20 of a disease in immediate family (parent, siblings, children), use of prescription  
drugs and/or reason therefor, length and/or number of hospital stays and  
exposure to environmental factors.
3. The subcollection of claim 1, wherein the symbology is a bar code.
4. A method of producing a database, comprising:  
25 identifying healthy members of a population;  
obtaining data comprising identifying information and obtaining historical  
information and data relating to the identified members of the population and  
their immediate family;  
entering the data into a database for each member of the population and  
30 associating the member and the data with an indexer.
5. The method of claim 4, further comprising:  
obtaining a body tissue or body fluid sample;

analyzing the body tissue or body fluid in the sample; and  
entering the results of the analysis for each member into the database  
and associating each result with the indexer representative of each member.

- 5       6. A database produced by the method of claim 4.
7. A database produced by the method of claim 5.
8. A database, comprising:  
          datapoints representative of a plurality of healthy organisms from  
whom biological samples are obtained,  
          wherein each datapoint is associated with data representative of  
10 the organism type and other identifying information.
9. The database of claim 8, wherein the datapoints are answers to  
questions regarding one or more of a parameters selected from the group  
consisting of ethnicity, age, gender, height, weight, alcohol intake, number of  
pregnancies, number of live births, vegetarians, type of physical activity, state of  
15 residence and/or length of residence in a particular state, educational level, age  
of parent at death, cause of parent death, former or current smoker, length of  
time as a smoker, frequency of smoking, occurrence of a disease in immediate  
family (parent, siblings, children), use of prescription drugs and/or reason  
therefor, length and/or number of hospital stays and exposure to environmental  
20 factors.
10. The database of claim 9, wherein the organisms are mammals and  
the samples are body fluids or tissues.
11. The database of claim 9, wherein the samples are selected from  
blood, blood fractions, cells and subcellular organelles.
- 25       12. The database of claim 8, further comprising,  
phenotypic data from an organism.
13. The database of claim 12, wherein the data includes one of physical  
characteristics, background data, medical data, and historical data.
14. The database of claim 8, further comprising,  
30 genotypic data from nucleic acid obtained from an organism.

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15. The database of claim 14, wherein genotypic data includes, genetic markers, non-coding regions, microsatellites, RFLPs, VNTRs, historical data of the organism, medical history, and phenotypic information.

16. The database of claim 8 that is a relational database.

5 17. The database of claim 16, wherein the data are related to an indexer datapoint representative of each organism from whom data is obtained.

18. A method of identifying polymorphisms that are candidate genetic markers, comprising:

identifying a polymorphism; and

10 identifying any pathway or gene linked to the locus of the polymorphism, wherein

the polymorphisms are identified in samples associated with a target population that comprises healthy subjects.

15 19. The method of claim 18, wherein the polymorphism is identified by detecting the presence of target nucleic acids in a sample by a method, comprising the steps of:

a) hybridizing a first oligonucleotide to the target nucleic acid;

b) hybridizing a second oligonucleotide to an adjacent region of the target nucleic acid;

20 c) ligating the hybridized oligonucleotides; and

c) detecting hybridized first oligonucleotide by mass spectrometry as an indication of the presence of the target nucleic acid.

20. The method of claim 18, wherein the polymorphism is identified by detecting target nucleic acids in a sample by a method, comprising the steps of:

25 a) hybridizing a first oligonucleotide to the target nucleic acid and hybridizing a second oligonucleotide to an adjacent region of the target nucleic acid;

b) contacting the hybridized first and second oligonucleotides with a cleavage enzyme to form a cleavage product; and

30 c) detecting the cleavage product by mass spectrometry as an indication of the presence of the target nucleic acid.

21. The method of claim 20 wherein the samples are from subjects in a healthy database.

22. The method of claim 18, wherein the polymorphism is identified by identifying target nucleic acids in a sample by primer oligo base extension  
5 (probe).

23. The method of 22, wherein primer oligo base extension, comprises:

- a) obtaining a nucleic acid molecule that contains a target nucleotide;
- b) optionally immobilizing the nucleic acid molecule onto a solid support,  
10 to produce an immobilized nucleic acid molecule;
- c) hybridizing the nucleic acid molecule with a primer oligonucleotide that is complementary to the nucleic acid molecule at a site adjacent to the target nucleotide;
- d) contacting the product of step c) with a composition comprising a  
15 dideoxynucleoside triphosphate or a 3'-deoxynucleoside triphosphates and a polymerase, so that only a dideoxynucleoside or 3'-deoxynucleoside triphosphate that is complementary to the target nucleotide is extended onto the primer; and
- e) detecting the extended primer, thereby identifying the target nucleotide.

20 24. The method of claim 23, wherein detection of the extended primer is effected by mass spectrometry, comprising:  
ionizing and volatilizing the product of step d) ; and  
detecting the extended primer by mass spectrometry, thereby identifying the target nucleotide.

25 25. The method of claim 24, wherein;  
samples are presented to the mass spectrometer as arrays on chips; and  
each sample occupies a volume that is about the size of the laser spot  
projected by the laser in a mass spectrometer used in matrix-assisted laser  
desorption/ionization (MALDI) spectrometry.

26. A combination, comprising:  
a database containing parameters associated with a datapoint representative of a subject from whom samples are obtained, wherein the subjects are healthy; and  
5 an indexed collection of the samples, wherein the index identifies the subject from whom the sample was obtained.
- 27 The combination of claim 26, wherein the parameter is selected from the group consisting of ethnicity, age, gender, height, weight, alcohol intake, number of pregnancies, number of live births, vegetarians, type of  
10 physical activity, state of residence and/or length of residence in a particular state, educational level, age of parent at death, cause of parent death, former or current smoker, length of time as a smoker, frequency of smoking, occurrence of disease in immediate family (parent, siblings, children), use of prescription drugs and/or reason therefor, length and/or number of hospital stays and  
15 exposure to environmental factors.
28. The combination of claim 26, wherein the database further contains genotypic data for each subject.
29. The combination of claim 26, wherein the samples are blood.
- 30 A data storage medium, comprising the database of claim 8.
- 20 31. A computer system, comprising the database of claim 8.
32. A system for high throughput processing of biological samples, comprising:  
a process line comprising a plurality of processing stations, each of which performs a procedure on a biological sample contained in a  
25 reaction vessel;  
a robotic system that transports the reaction vessel from processing station to processing station;  
a data analysis system that receives test results of the process line and automatically processes the test results to make a determination  
30 regarding the biological sample in the reaction vessel;  
a control system that determines when the test at each processing station is complete and, in response, moves the reaction vessel to

the next test station, and continuously processes reaction vessels one after another until the control system receives a stop instruction; and

5 a database of claim 8, wherein the samples tested by the automated process line comprise samples from subjects in the database.

33. The system of claim 32, wherein one of the processing stations comprises a mass spectrometer.

34. The system of claim 32, wherein the data analysis system processes the test results by receiving test data from the mass spectrometer  
10 such that the test data for a biological sample contains one or more signals, whereupon the data analysis system determines the area under the curve of each signal and normalizes the results thereof and obtains a substantially quantitative result representative of the relative amounts of components in the tested sample.

15 35. A method for high throughput processing of biological samples, the method comprising:

transporting a reaction vessel along a system of claim 32, comprising a process line having a plurality of processing stations, each of which performs a procedure on one or more biological samples  
20 contained in the reaction vessel;  
determining when the test procedure at each processing station is complete and, in response, moving the reaction vessel to the next processing station;  
receiving test results of the process line and automatically processing the  
25 test results to make a data analysis determination regarding the biological samples in the reaction vessel; and  
processing reaction vessels continuously one after another until receiving a stop instruction, wherein the samples tested by the automated process line comprise samples from subjects in the database.

30 36. The method of 35, wherein one of the processing stations comprises a mass spectrometer.

37. The method of claim 36, wherein the samples are analyzed by a method comprising primer oligo base extension (probe).

38. The method of claim 37, further comprising:

processing the test results by receiving test data from the mass spectrometer such that the test data for a biological sample contains one or more signals or numerical values representative of signals, whereupon the data analysis system determines the area under the curve of each signal and normalizes the results thereof and obtains a substantially quantitative result representative of the relative amounts of components in the tested sample.

39. The method of claim 37, wherein primer oligo base extension, comprises:

- a) obtaining a nucleic acid molecule that contains a target nucleotide;
- b) optionally immobilizing the nucleic acid molecule onto a solid support, to produce an immobilized nucleic acid molecule;
- c) hybridizing the nucleic acid molecule with a primer oligonucleotide that is complementary to the nucleic acid molecule at a site adjacent to the target nucleotide;
- d) contacting the product of step c) with composition comprising a dideoxynucleoside triphosphate or a 3'-deoxynucleoside triphosphates and a polymerase, so that only a dideoxynucleoside or 3'-deoxynucleoside triphosphate that is complementary to the target nucleotide is extended onto the primer; and
- e) detecting the primer, thereby identifying the target nucleotide.

40. The method of 39, wherein detection of the extended primer is effected by mass spectrometry, comprising:  
ionizing and volatilizing the product of step d); and  
detecting the extended primer by mass spectrometry, thereby identifying the target nucleotide.

41. The method of claim 36, wherein the target nucleic acids in the sample are detected and/or identified by a method, comprising the steps of:

- a) hybridizing a first oligonucleotide to the target nucleic acid;
- b) hybridizing a second oligonucleotide to an adjacent region of the target nucleic acid;

c) ligating then hybridized oligonucleotides; and  
 c) detecting hybridized first oligonucleotide by mass spectrometry as an indication of the presence of the target nucleic acid.

42. The method of claim 36, wherein the target nucleic acids in the  
 5 sample are detected and/or identified by a method, comprising the steps of:

a) hybridizing a first oligonucleotide to the target nucleic acid and hybridizing a second oligonucleotide to an adjacent region of the target nucleic acid;

b) contacting the hybridized first and second oligonucleotides with a  
 10 cleavage enzyme to form a cleavage product; and

c) detecting the cleavage product by mass spectrometry as an indication of the presence of the target nucleic acid.

43. A method of producing a database stored in a computer memory, comprising:

15 identifying healthy members of a population;

obtaining identifying and historical information and data relating to the identified members of the population;

entering the member-related data into the computer memory database for each identified member of the population and associating the member and the  
 20 data with an indexer.

44. The method of claim 43, further comprising:

obtaining a body tissue or body fluid sample of an identified member;

analyzing the body tissue or body fluid in the sample; and

entering the results of the analysis for each member into the computer  
 25 memory database and associating each result with the indexer representative of each member.

45. A database produced by the method of claim 43.

46. A database produced by the method of claim 44.

47. The database of claim 8, wherein:

30 the organisms are selected from among animals, bacteria, fungi, protozoans and parasites and

each datapoint is associated with parameters representative of the organism type and identifying information.

48. The database of claim 43, further comprising, phenotypic data regarding each subject.

5 49. The database of claim 47 that is a relational database and the parameters are the answers to the questions in the questionnaire.

50. The database of claim 8, further comprising, genotypic data of nucleic acid of the subject, wherein genotypic data includes, but is not limited to, genetic markers, non-coding regions,  
10 microsatellites, restriction fragment length polymorphisms (RFLPs), variable number tandem repeats (VNTRs), historical day of the organism, the medical history of the subject, phenotypic information, and other information.

51. A database, comprising data records stored in computer memory, wherein the data records contain information that identifies healthy members of  
15 a population, and also contain identifying and historical information and data relating to the identified members.

52. The database of claim 51, further comprising an index value for each identified member that associates each member of the population with the identifying and historical information and data.

20 53. A computer system, comprising the database of claim 51.

54. An automated process line, comprising the database of claim 51.

55. A method for determining a polymorphism that correlates with age, ethnicity or gender, comprising:

identifying a polymorphism; and  
25 determining the frequency of the polymorphism with increasing age, with ethnicity or with gender in a healthy population.

56. A method for determining whether a polymorphism correlates with susceptibility to morbidity, early mortality, or morbidity and early mortality, comprising:

30 identifying a polymorphism; and  
determining the frequency of the polymorphism with increasing age in a healthy population.

57. A high throughput method of determining frequencies of genetic variations, comprising:

selecting a healthy target population and a genetic variation to be assessed;

5 pooling a plurality of samples of biopolymers obtained from members of the population,

determining or detecting the biopolymer that comprises the variation by mass spectrometry;

10 obtaining a mass spectrum or a digital representation thereof; and  
determining the frequency of the variation in the population.

58. The method of claim 57, wherein:

the variation is selected from the group consisting of an allelic variation, a post-translational modification, a nucleic modification, a label, a mass modification of a nucleic acid and methylation; and/or

15 the biopolymer is a nucleic acid, a protein, a polysaccharide, a lipid, a small organic metabolite or intermediate, wherein the concentration of biopolymer of interest is the same in each of the samples; and/or

the frequency is determined by assessing the method comprising determining the area under the peak in the mass spectrum or digital

20 representation thereof corresponding to the mass of the biopolymer comprising the genomic variation.

59. The method of claim 58, wherein the method for determining the frequency is effected by determining the ratio of the signal or the digital representation thereof to the total area of the entire mass spectrum, which is  
25 corrected for background.

60. A method for discovery of a polymorphism in a population, comprising:

sorting the database of claim 8 according to a selected parameter to identify samples that match the selected parameter;

30 isolating a biopolymer from each identified sample;

optionally pooling each isolated biopolymer;

optionally amplifying the amount of biopolymer;

cleaving the pooled biopolymers to produce fragments thereof;  
obtaining a mass spectrum of the resulting fragments and comparing the  
mass spectrum with a control mass spectrum to identify differences between the  
spectra and thereby identifying any polymorphisms; wherein:

5 the control mass spectrum is obtained from unsorted samples in the  
collection or samples sorted according to a different parameter.

61. The method of claim 60, wherein cleaving is effected by contacting  
the biopolymer with an enzyme.

62. The method of claim 61, wherein the enzyme is selected from the  
10 group consisting of nucleotide glycosylase, a nickase and a type IIS restriction  
enzyme.

63. The method of claim 60, wherein the biopolymer is a nucleic acid  
or a protein.

64. The method of claim 60, wherein the the mass spectrometric  
15 format is selected from among Matrix-Assisted Laser Desorption/Ionization,  
Time-of-Flight (MALDI-TOF), Electrospray (ES), IR-MALDI, Ion Cyclotron  
Resonance (ICR), Fourier Transform and combinations thereof.

65. A method for discovery of a polymorphism in a population,  
comprising:

20 obtaining samples of body tissue or fluid from a plurality of organisms;  
isolating a biopolymer from each sample;  
pooling each isolated biopolymer;  
optionally amplifying the amount of biopolymer;  
cleaving the pooled biopolymers to produce fragments thereof;  
25 obtaining a mass spectrum of the resulting fragments;  
comparing the frequency of each fragment to identify fragments present  
in amounts lower than the average frequency, thereby identifying any  
polymorphisms.

66. The method of claim 65, wherein cleaving is effected by contacting  
30 the biopolymer with an enzyme.

67. The method of claim 66, wherein the enzyme is selected from the group consisting of nucleotide glycosylase, a nickase and a type IIS restriction enzyme.

68. The method of claim 65, wherein the biopolymer is a nucleic acid  
5 or a protein.

69. The method of claim 65, wherein the the mass spectrometric format is selected from among Matrix-Assisted Laser Desorption/Ionization, Time-of-Flight (MALDI-TOF), Electrospray (ES), IR-MALDI, Ion Cyclotron Resonance (ICR), Fourier Transform and combinations thereof.

10 70. The method of claim 65, wherein the samples are obtained from healthy subjects.

71. A method of correlating a polymorphism with a parameter, comprising:

15 sorting the database of claim 8 according to a selected parameter to identify samples that match the selected parameter;

isolating a biopolymer from each identified sample;

pooling each isolated biopolymer;

optionally amplifying the amount of biopolymer;

determining the frequency of the polymorphism in the pooled

20 biopolymers, wherein:

an alteration of the frequency of the polymorphism compared to a control, indicates a correlation of the polymorphism with the selected parameter; and

the control is the frequency of the polymorphism in pooled biopolymers obtained from samples identified from an unsorted database or from a database  
25 sorting according to a different parameter.

72. The method claim 71, wherein the parameter is selected from the group consisting of ethnicity, age, gender, height, weight, alcohol intake, number of pregnancies, number of live births, vegetarians, type of physical activity, state of residence and/or length of residence in a particular state,  
30 educational level, age of parent at death, cause of parent death, former or current smoker, length of time as a smoker, frequency of smoking, occurrence of a disease in immediate family (parent, siblings, children), use of prescription

73. The method claim 72, wherein the parameter is occurrence of disease or a particular disease in an immediate family member, thereby correlating the polymorphism with the disease.

75. The method of claim 74, wherein the polymorphism is detected by primer oligo base extension (PROBE).

a) optionally immobilizing the nucleic acid molecules onto a solid support, to produce immobilized nucleic acid molecules;

c) contacting the product of step c) with composition comprising a dideoxynucleoside triphosphate or a 3'-deoxynucleoside triphosphates and a polymerase, so that only a dideoxynucleoside or 3'-deoxynucleoside triphosphate

d) detecting the extended primer, thereby detecting the polymorphism in nucleic acid molecules in the pooled nucleic acids.

78. The method of claim 71, wherein the frequency is percentage of nucleic acid molecules in the pooled nucleic acids that contain the polymorphism.

80. The method of claim 72, wherein the parameter is age, thereby correlating the polymorphism with susceptibility to morbidity, early mortality or morbidity and early mortality.

81. A method for haplotyping polymorphisms in a nucleic acid, comprising:

- (a) sorting the database of claim 8 according to a selected parameter to identify samples that match the selected parameter;
- 5 (b) isolating nucleic acid from each identified sample;
- (c) optionally pooling each isolated nucleic acid;
- (d) amplifying the amount of nucleic acid;
- (e) forming single-stranded nucleic acid and splitting each single-strand into a separate reaction vessel;
- 10 (f) contacting each single-stranded nucleic acid with an adaptor nucleic acid to form an adaptor complex;
- (g) contacting the adaptor complex with a nuclease and a ligase;
- (h) contacting the products of step (g) with a mixture that is capable of amplifying a ligated adaptor to produce an extended product;
- 15 (i) obtaining a mass spectrum of each nucleic acid resulting from step (h) and detecting a polymorphism by identifying a signal corresponding to the extended product;
- (j) repeating steps (f) through (i) utilizing an adaptor nucleic acid able to hybridize with another adapter nucleic acid that hybridizes to a different
- 20 sequence on the same strand; whereby  
the polymorphisms are haplotyped by detecting more than one extended product.

82. The method of claim 1, wherein the nuclease is Fen-1.

- 83. A method for haplotyping polymorphisms in a population, comprising:
- sorting the database of claim 8 according to a selected parameter to identify samples that match the selected parameter;
- isolating a nucleic acid from each identified sample;
- pooling each isolated nucleic acid;
- 30 optionally amplifying the amount of nucleic acid;
- contacting the nucleic acid with at least one enzyme to produce fragments thereof;

obtaining a mass spectrum of the resulting fragments; whereby:  
the polymorphisms are detected by detecting signals corresponding to the  
polymorphisms; and

the polymorphisms are haplotyped by determining from the mass  
5 spectrum that the polymorphisms are located on the same strand of the nucleic  
acid.

84. The method of claim 83, wherein the enzyme is a nickase.

85. The method of claim 84, wherein the nickase is selected from the  
group consisting of NY2A and NYS1.

10 86. A method for detecting methylated nucleotides within a nucleic  
acid sample, comprising:

splitting a nucleic acid sample into separate reaction vessels;

contacting nucleic acid in one reaction vessel with bisulfite;

amplifying the nucleic acid in each reaction vessel;

15 cleaving the nucleic acids in each reaction vessel to produce fragments  
thereof;

obtaining a mass spectrum of the resulting fragments from one reaction  
vessel and another mass spectrum of the resulting fragments from another  
reaction vessel; whereby:

20 cytosine methylation is detected by identifying a difference in signals  
between the mass spectra.

87. The method of claim 86, wherein:

the step of amplifying is carried out in the presence of uracil; and

the step of cleaving is effected by a uracil glycosylase.

25 88. A method for identifying a biological sample, comprising:  
generating a data set indicative of the composition of the biological  
sample;

denoising the data set to generate denoised data;

deleting the baseline from the denoised data to generate an intermediate

30 data set;

defining putative peaks for the biological sample;

using the putative peaks to generate a residual baseline;

removing the residual baseline from the intermediate data set to generate a corrected data set;

locating, responsive to removing the residual baseline, a probable peak in the corrected data set; and

- 5 identifying, using the located probable peak, the biological sample;  
wherein the generated biological sample data set comprises data from sense strands and antisense strands of assay fragments.

89. The method according to claim 88, wherein identifying includes combining data from the sense strands and the antisense strands, and  
10 comparing the data against expected sense strand and antisense strand values, to identify the biological sample.

90. The method according to claim 88, wherein identifying includes deriving a peak probability for the probable peak, in accordance with whether the  
15 probable peak is from sense strand data or from antisense strand data.

91. The method according to claim 88, wherein identifying includes deriving a peak probability for the probable peak and applying an allelic penalty in response to a ratio between a calculated area under the probable peak and a calculated expected average area under all peaks in the data set.

- 20 92. A method for identifying a biological sample, comprising:  
generating a data set indicative of the composition of the biological sample;

denoising the data set to generate denoised data;

- 25 deleting the baseline from the denoised data to generate an intermediate data set;

defining putative peaks for the biological sample; using the putative peaks to generate a residual baseline;

removing the residual baseline from the intermediate data set to generate a corrected data set;

- 30 locating, responsive to removing the residual baseline, a probable peak in the corrected data set; and

identifying, using the located probable peak, the biological sample;  
wherein identifying includes deriving a peak probability for the probable  
peak and

applying an allelic penalty in response to a ratio between a calculated  
5 area under the probable peak and a calculated expected average area under all  
peaks in the data set.

93. The method according to claim 92, wherein identifying includes  
comparing data from probable peaks that did not receive an applied allelic  
penalty to determine their mass in accordance with oligonucleotide biological  
10 data.

94. The method according to claim 92, wherein the allelic penalty is  
not applied to probable peaks whose ratio of area under the peak to the  
expected area value is greater than 30%.

95. A method for detecting a polymorphism in a nucleic acid,  
15 comprising:  
amplifying a region of the nucleic acid to produce an amplicon, wherein  
the resulting amplicon comprises one or more enzyme restriction sites;  
contacting the amplicon with a restriction enzyme to produce fragments;  
obtaining a mass spectrum of the resulting fragments and analyzing  
20 signals in the mass spectrum by the method of claim 88; whereby:  
the polymorphism is detected from the pattern of the signals.

96. A subcollection of samples from a target population, comprising:  
a plurality of samples, wherein the samples are selected from the group  
consisting of nucleic acids, fetal tissue, protein samples; and  
25 a symbology on the containers containing the samples, wherein the  
symbology is representative of the source and/or history of each sample,  
wherein:

the target population is a healthy population that has not been selected  
for any disease state;  
30 the collection comprises samples from the healthy population; and  
the subcollection is obtained by sorting the collection according to  
specified parameters.

97. The combination of claim 26, wherein the samples are selected selected from the group consisting of nucleic acids, fetal tissue, protein, tissue, body fluid, cell, seed, microbe, pathogen and reproductive tissue samples.

98. A combination, comprising the database of claim 8 and a mass  
5 spectrometer.

99. The combination of claim 98 that is an automated process line for analyzing biological samples.

100. A system for high throughput processing of biological samples, comprising:

10 a database of claim 8, wherein the samples tested by the automated process line comprise samples from subjects in the database; and a mass spectrometry for analysis of biopolymers in the samples.

**ABSTRACT**

Process and methods for creating a database of genomic samples from healthy human donors, methods that use the database to identify and correlate polymorphic genetic markers and other markers with diseases and conditions are  
5 provided.

09687483-101300

005101F\*EBH78960

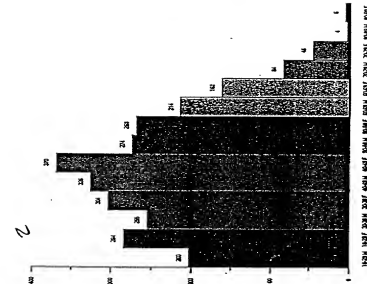
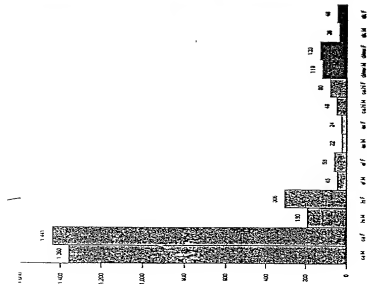
# Sample Bank



DNA Bank

Caucasians

Hispanics



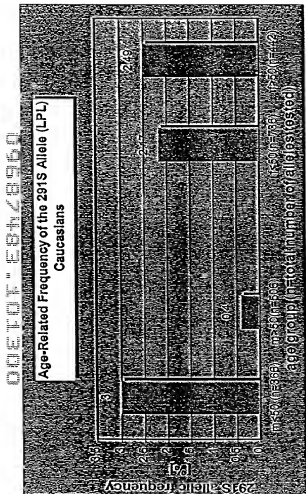


FIGURE 2A

Age- and sex-distribution of the 291S allele of the lipoprotein lipase gene. A total of 436 males and 589 females were investigated.

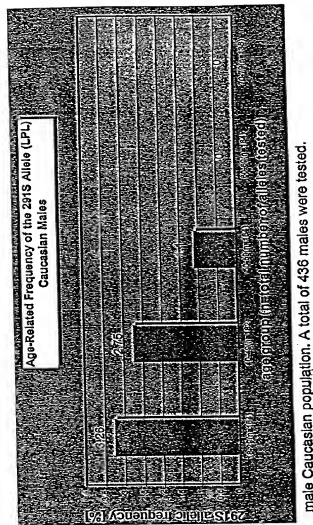
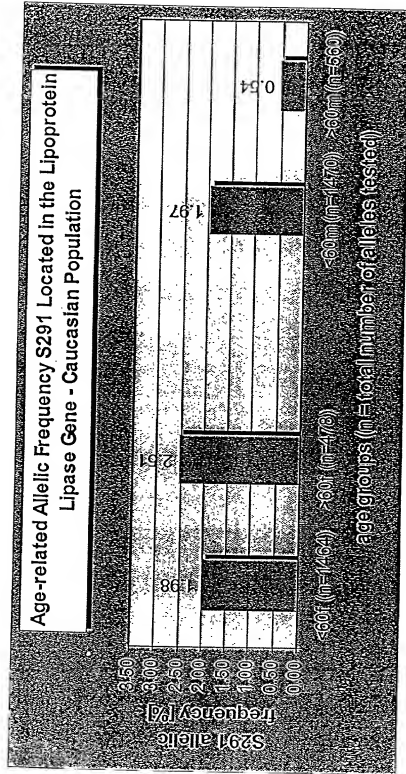


FIGURE 2B

male Caucasian population. A total of 436 males were tested.



Significance: Allele frequency of Ser291 drops from 1.97% to 0.54% in males;  $p=0.009$

FIGURE 2C



00E1D1"E84Z8960

# Intelligent Genomics



## Sample Banks

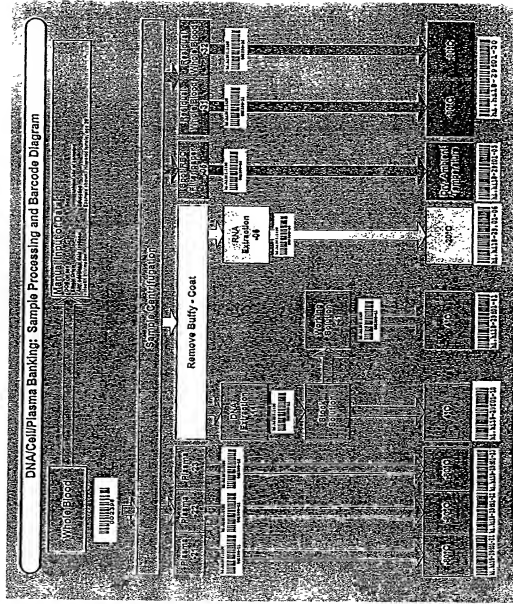


FIGURE 4

# Intelligent Genomics



Age-Related Distribution of "Sick" and "Healthy" Alleles in a  
"Healthy" Population

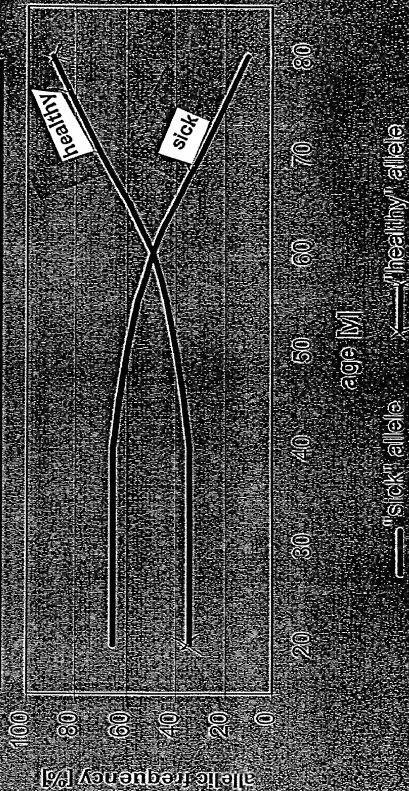


FIGURE 5

# Intelligent Genomics



Age-Dependent Distribution of ApoE Genotypes / Alleles

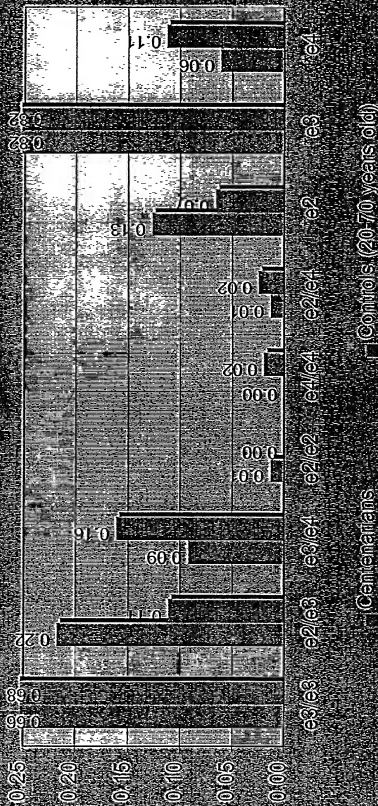
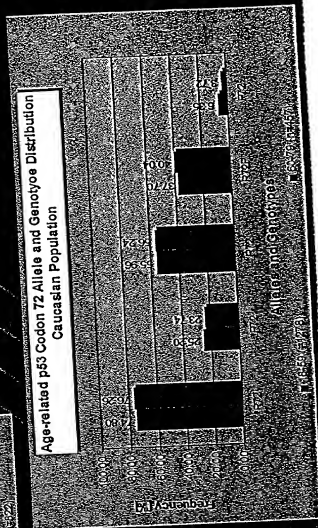
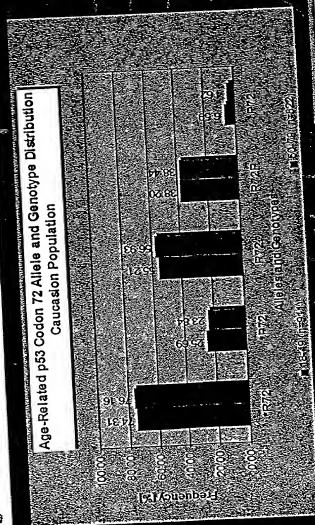


FIGURE 6



FIGURE 7A

i genomics



i genomics

Age-Related p53 Codon 72 Allele Distribution  
Caucasian Population

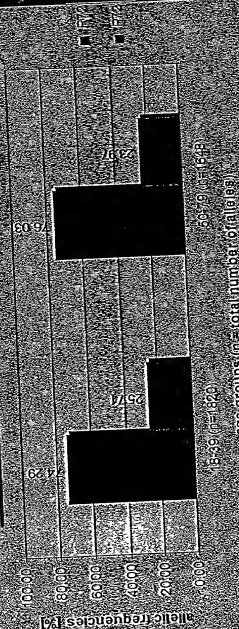
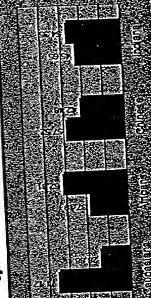


FIGURE 7B

Allele Distribution among Different Ethnic Groups  
Sjlander et al. Hum Hered 46: 41-48, 1996





SEQUENOM  
INDUSTRIAL GENOMICS

FIGURE 7C

i genetics

# P53 PP vs. PR/RR Genotype Distribution By Age cut point = 59

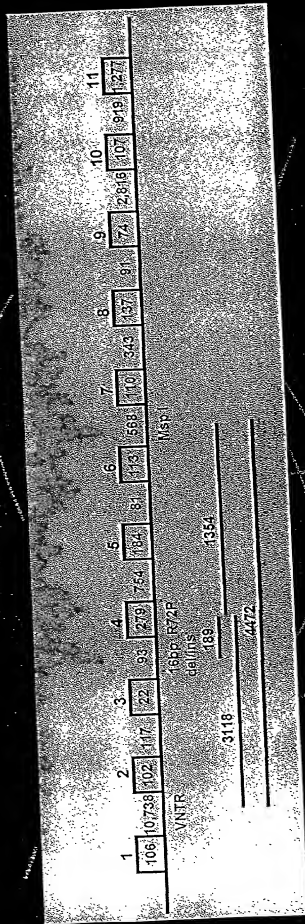
Age Group	Genotype Freq (%)		
	N	PP	PR/RR
18-59	1278	6.7	93.4
60-79	457	3.7	96.3

Sample Size : 1735

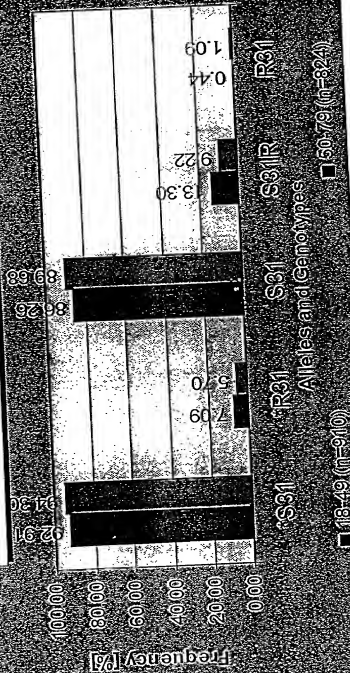
$p = 0.02$

FIGURE 7D

# Genomic Organization of the p53 Gene



# Age-Related p21 S31R Allele and Genotype Frequencies Caucasian Population



Significance: Genotype frequency of SR heterozygous drops from 13.3% to 9.2%;  $p=0.009$

00E101-2B478960



SEQUENOM  
INDUSTRIAL GENOMICS

DNA MassArray™

FVII R353Q

Allelic Frequency in Pooled vs Individual Samples  
FVII Allele 353Q

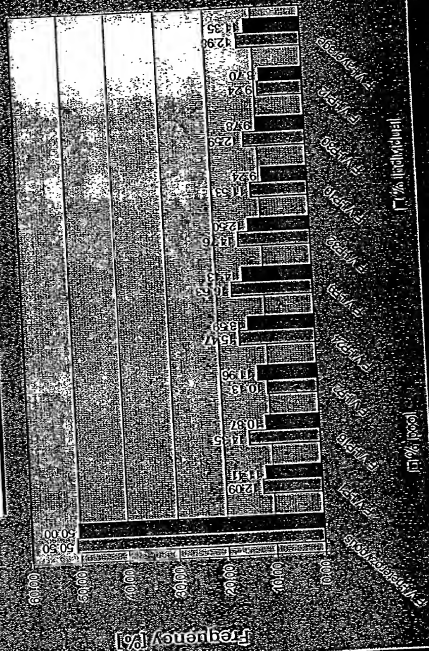


FIGURE 9

00EFDY\*E8H78960

DNA MassArray™

CETP I405V



Allelic Frequency in Pooled vs Individual Samples  
CETP Allele 405V

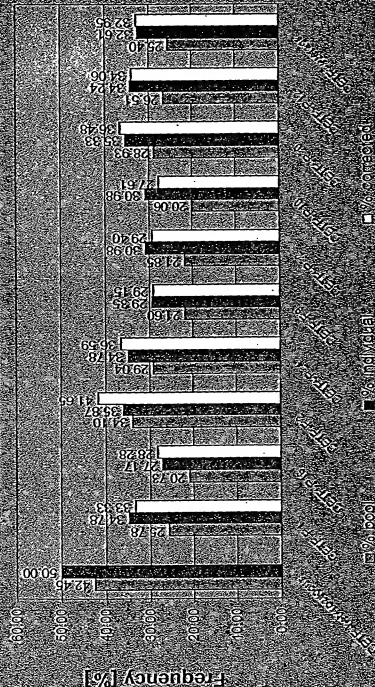


FIGURE 10

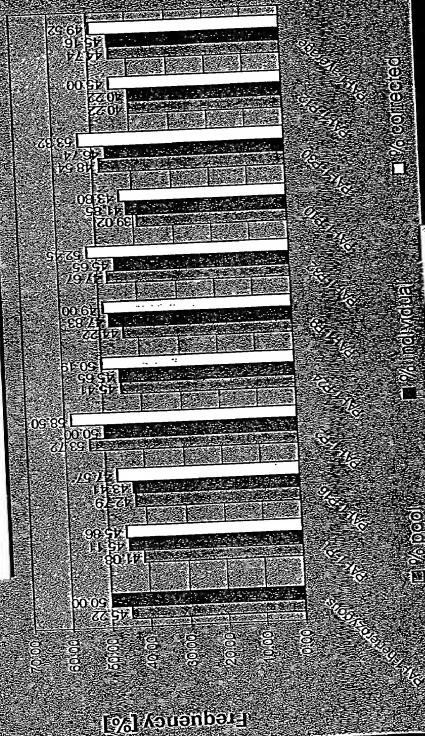
002101" E8h78960



**DNA MassArray™**

**PAI-1 4G/5G**

**Allelic Frequency in Pooled vs Individual Samples  
Allele PAI-1 5G**



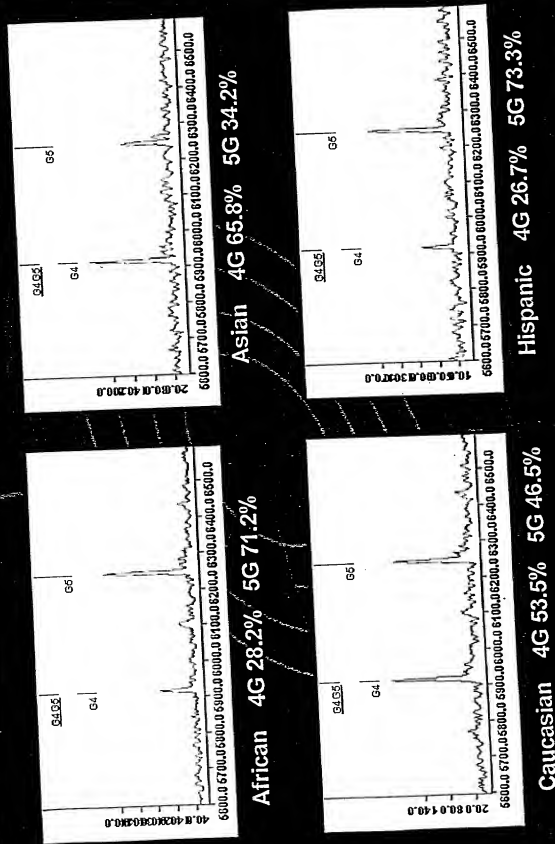
**FIGURE 11**



# DNA MassArray™

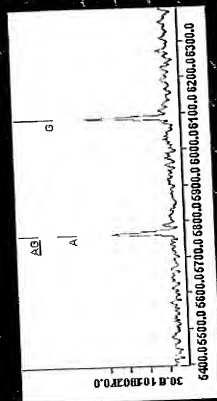
## Ethnic Diversity (PAL-1)

FIGURE 12

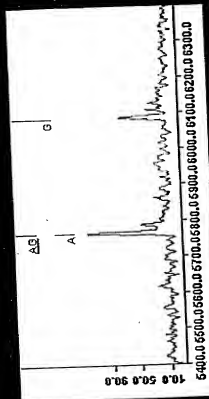


# DNA MassArray™

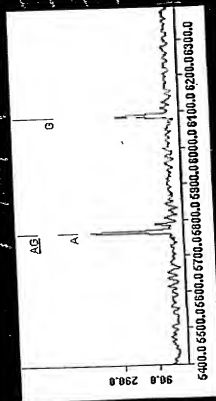
## Ethnic Diversity (CETP 405)



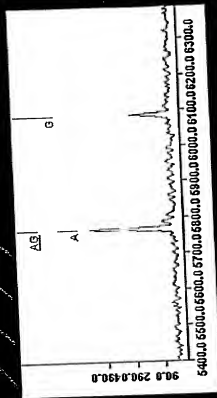
African Ile 43.6% Val 56.4%



Asian Ile 60.7% Val 39.3%



Caucasian Ile 63.0% Val 37.0%

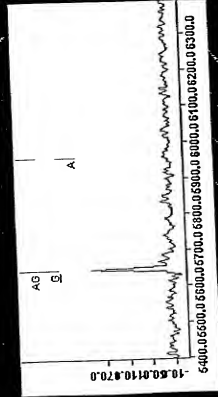


Hispanic Ile 70.1% Val 29.9%

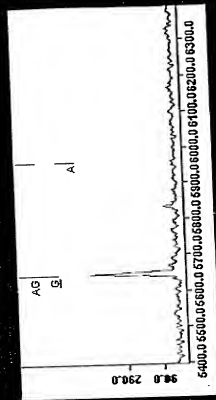
FIGURE 13

## DNA MassArray™

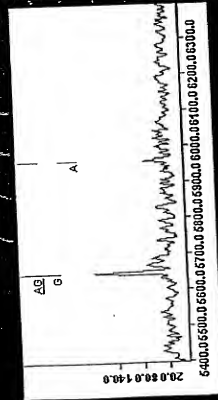
Ethnic Diversity (Factor VII 353)



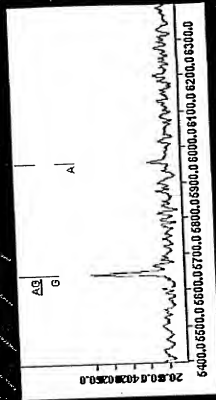
African Arg &gt;95% Glu &lt;5%



Asian Arg &gt;95% Glu &lt;5%



Caucasian Arg 88.5% Glu 11.5%



Hispanic Arg 89.2% Glu 10.8%

FIGURE 14

## DNA MassArray™

## Ethnic Diversity



Ethnic diversity using pooled DNA samples

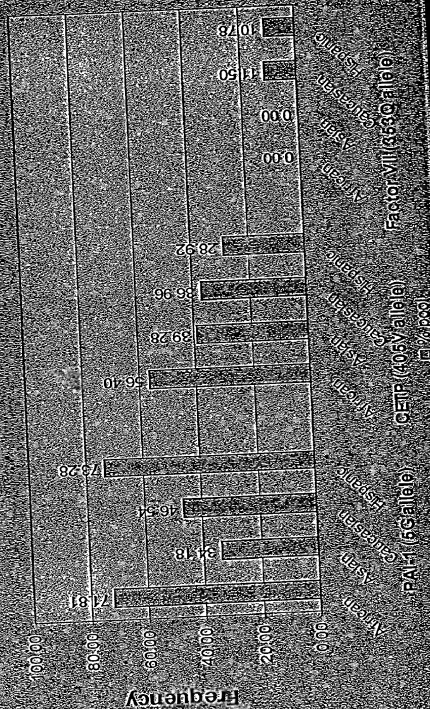
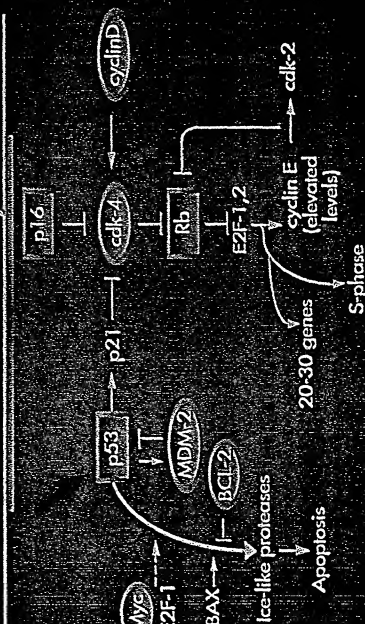


FIGURE 15

1997-02-28

# P53-Rb Pathway



09687483 101300

1700

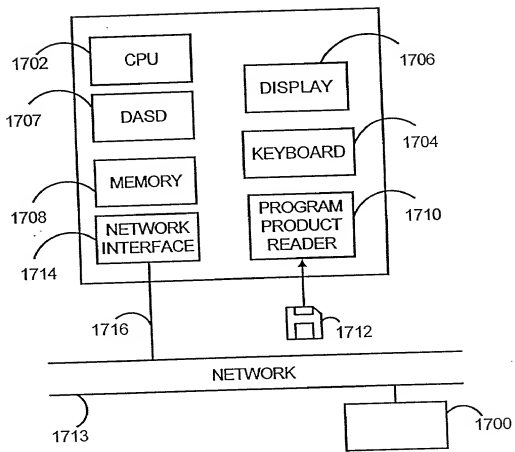


FIGURE 17

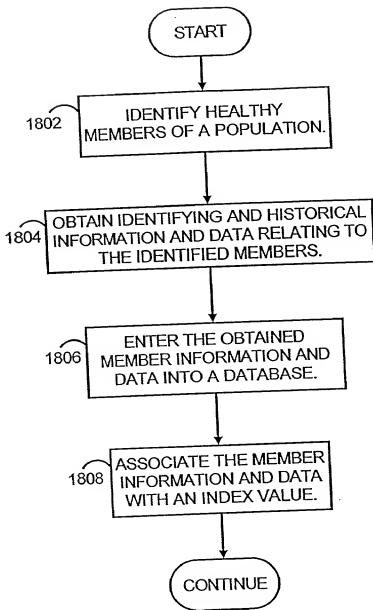


FIGURE 18

# clone chromosome 17 (#48319)

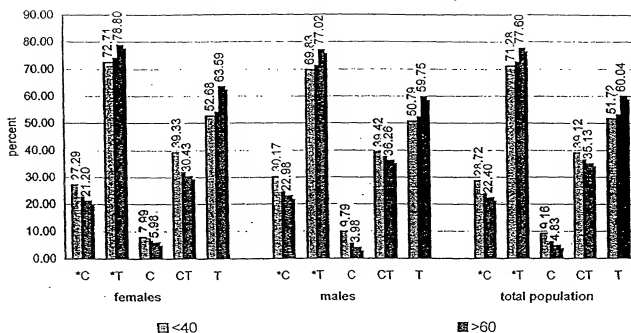


Figure 19

00667483.101300

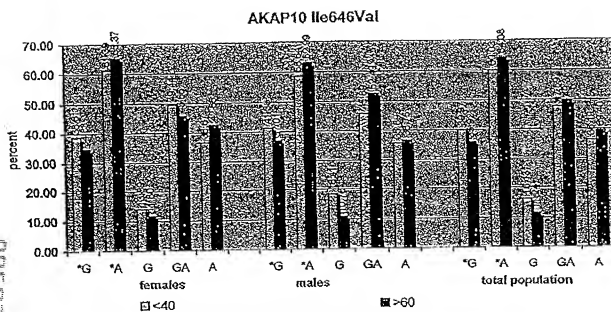
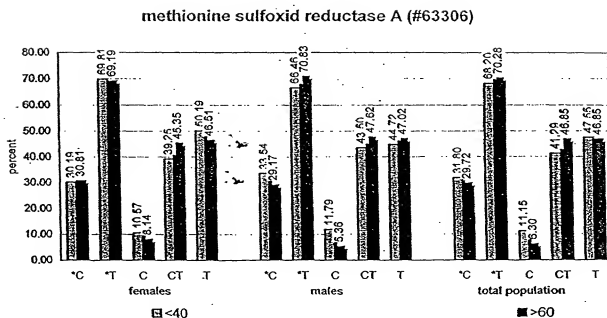


Figure 20

Figure 21



**FIGURE 22A**

**3** In which state do you live?

A	A
B	B
C	C
D	D
E	E
F	F
G	G
H	H
I	I
J	J
K	K
L	L
M	M
N	N
O	O
P	P
Q	Q
R	R
S	S
T	T
U	U
V	V
W	W
X	X
Y	Y
Z	Z



# Collection Information

Consent Form Signed

☐ Yes ☐ No

Time of Sample Collection (nearest hour, in 24 hour clock format)

Initials

Initials of Data Collector

Date of Collection		
Month	Day	Year
JAN	1	00
FEB	2	00
MAR	3	00
APR	4	00
MAY	5	00
JUN	6	00
JUL	7	00
AUG	8	00
SEP	9	00
OCT	10	00
NOV	11	00
DEC	12	00

Initials
00 00
01 00
02 00
03 00
04 00
05 00
06 00
07 00
08 00
09 00
10 00
11 00
12 00
13 00
14 00
15 00
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(DO NOT COMPLETE for data entry only)

Sample	Volume (ml)
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Bar Code

## Donor Information

Date of Birth	
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Sex:

☐ Male ☐ Female

Height (inches)
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What physical activity do you do on a regular basis?

☐ Running  
☐ Swimming  
☐ Biking  
☐ Gymnastics  
☐ Other  
☐ None

Are you a vegetarian?

☐ Yes ☐ No

If Female:

How many times have you been pregnant?

How many times did you give birth?

In which state do you live?

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To the best of your knowledge, what is the Ethnic Origin of you:

Father Mother

☐ Caucasian (please mark specific geographic area below if known)  
☐ Northern Europe (Austria, Denmark, Finland, France, Germany, Netherlands, Norway, Sweden, Switzerland, UK)  
☐ Southern Europe (Greece, Italy, Spain, Turkey)  
☐ Eastern Europe (Czechoslovakia, Hungary, Poland, Russia, Yugoslavia)  
☐ Middle Eastern (Israel, Egypt, Iran, Iraq, Jordan, Syria, Other Arab States)  
☐ Other  
☐ Don't know

How long have you lived there?

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How many years have you been smoking?

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Did you quit smoking?

☐ Yes ☐ No

If yes, how many years ago?

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How many cigarettes did you smoke per day?

Cigarettes
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at completed in school?  
 1 less than 8th grade  
 1 8th, 9th, 10th, or 11th grade  
 1 high school graduate or  
 equivalent  
 1 some college, 2 yr degree  
 1 college graduate, 4 yr degree  
 1 post graduate education or  
 degree

☐ Yes  
☐ No  
 If Yes at  
 what age?

☐ < 29  
☐ 30-39  
☐ 40-49  
☐ 50-59  
☐ 60-69  
☐ 70-79  
☐ 80-89  
☐ ≥ 90

☐ Heart Disease  
☐ Cancer  
☐ Stroke  
☐ Accident  
☐ Suicide  
☐ Other

☐ Yes  
☐ No  
 If Yes at  
 what age?

☐ < 29  
☐ 30-39  
☐ 40-49  
☐ 50-59  
☐ 60-69  
☐ 70-79  
☐ 80-89  
☐ ≥ 90

☐ Heart Disease  
☐ Cancer  
☐ Stroke  
☐ Accident  
☐ Suicide  
☐ Other

## Health Information

Do you or has anyone in your immediate family (parents, brothers, sisters, or your children) had the following?  
 Please specify:

	You	Mother	Father	Sister	Brother	Child
Heart Disease	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Stroke	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Hypertension	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Abnormal blood clots	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Diabetes, insulin dependent	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Diabetes, not insulin-dependent (diet controlled)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Cancer	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Lung & Bronchus	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Breasts	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Prostate	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Colon & Rectum	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Skin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Lymphoma & Leukemia	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Other, please specify below:	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Alzheimer's Disease	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Epilepsy	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Schizophrenia	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Bipolar disorder (manic depression)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Major depression	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Chronic Inflammatory or Autoimmune Disease including Multiple Sclerosis and Rheumatoid Arthritis	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Emphysema	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Asthma	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Other, please specify below:	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Do you take prescription drugs on a regular basis?

☐ Yes, please specify below:

☐ Yes ☐ No

Have you ever donated blood before? ☐ Yes ☐ No

If yes, how many times: Number of Times

<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
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<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>

Have you been hospitalized in the past 5 years for more than 6 days at a time?  
☐ Yes ☐ No

If yes, how many times?

1) Weeks: ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8 ☐ 9 ☐ 10 ☐ 11 ☐ 12 ☐ 13 ☐ 14 ☐ 15 ☐ 16 ☐ 17 ☐ 18 ☐ 19 ☐ 20 ☐ 21 ☐ 22 ☐ 23 ☐ 24 ☐ 25 ☐ 26 ☐ 27 ☐ 28 ☐ 29 ☐ 30 ☐ 31 ☐ 32 ☐ 33 ☐ 34 ☐ 35 ☐ 36 ☐ 37 ☐ 38 ☐ 39 ☐ 40 ☐ 41 ☐ 42 ☐ 43 ☐ 44 ☐ 45 ☐ 46 ☐ 47 ☐ 48 ☐ 49 ☐ 50 ☐ 51 ☐ 52 ☐ 53 ☐ 54 ☐ 55 ☐ 56 ☐ 57 ☐ 58 ☐ 59 ☐ 60 ☐ 61 ☐ 62 ☐ 63 ☐ 64 ☐ 65 ☐ 66 ☐ 67 ☐ 68 ☐ 69 ☐ 70 ☐ 71 ☐ 72 ☐ 73 ☐ 74 ☐ 75 ☐ 76 ☐ 77 ☐ 78 ☐ 79 ☐ 80 ☐ 81 ☐ 82 ☐ 83 ☐ 84 ☐ 85 ☐ 86 ☐ 87 ☐ 88 ☐ 89 ☐ 90 ☐ 91 ☐ 92 ☐ 93 ☐ 94 ☐ 95 ☐ 96 ☐ 97 ☐ 98 ☐ 99 ☐ 100 ☐ 101 ☐ 102 ☐ 103 ☐ 104 ☐ 105 ☐ 106 ☐ 107 ☐ 108 ☐ 109 ☐ 110 ☐ 111 ☐ 112 ☐ 113 ☐ 114 ☐ 115 ☐ 116 ☐ 117 ☐ 118 ☐ 119 ☐ 120 ☐ 121 ☐ 122 ☐ 123 ☐ 124 ☐ 125 ☐ 126 ☐ 127 ☐ 128 ☐ 129 ☐ 130 ☐ 131 ☐ 132 ☐ 133 ☐ 134 ☐ 135 ☐ 136 ☐ 137 ☐ 138 ☐ 139 ☐ 140 ☐ 141 ☐ 142 ☐ 143 ☐ 144 ☐ 145 ☐ 146 ☐ 147 ☐ 148 ☐ 149 ☐ 150 ☐ 151 ☐ 152 ☐ 153 ☐ 154 ☐ 155 ☐ 156 ☐ 157 ☐ 158 ☐ 159 ☐ 160 ☐ 161 ☐ 162 ☐ 163 ☐ 164 ☐ 165 ☐ 166 ☐ 167 ☐ 168 ☐ 169 ☐ 170 ☐ 171 ☐ 172 ☐ 173 ☐ 174 ☐ 175 ☐ 176 ☐ 177 ☐ 178 ☐ 179 ☐ 180 ☐ 181 ☐ 182 ☐ 183 ☐ 184 ☐ 185 ☐ 186 ☐ 187 ☐ 188 ☐ 189 ☐ 190 ☐ 191 ☐ 192 ☐ 193 ☐ 194 ☐ 195 ☐ 196 ☐ 197 ☐ 198 ☐ 199 ☐ 200 ☐ 201 ☐ 202 ☐ 203 ☐ 204 ☐ 205 ☐ 206 ☐ 207 ☐ 208 ☐ 209 ☐ 210 ☐ 211 ☐ 212 ☐ 213 ☐ 214 ☐ 215 ☐ 216 ☐ 217 ☐ 218 ☐ 219 ☐ 220 ☐ 221 ☐ 222 ☐ 223 ☐ 224 ☐ 225 ☐ 226 ☐ 227 ☐ 228 ☐ 229 ☐ 230 ☐ 231 ☐ 232 ☐ 233 ☐ 234 ☐ 235 ☐ 236 ☐ 237 ☐ 238 ☐ 239 ☐ 240 ☐ 241 ☐ 242 ☐ 243 ☐ 244 ☐ 245 ☐ 246 ☐ 247 ☐ 248 ☐ 249 ☐ 250 ☐ 251 ☐ 252 ☐ 253 ☐ 254 ☐ 255 ☐ 256 ☐ 257 ☐ 258 ☐ 259 ☐ 260 ☐ 261 ☐ 262 ☐ 263 ☐ 264 ☐ 265 ☐ 266 ☐ 267 ☐ 268 ☐ 269 ☐ 270 ☐ 271 ☐ 272 ☐ 273 ☐ 274 ☐ 275 ☐ 276 ☐ 277 ☐ 278 ☐ 279 ☐ 280 ☐ 281 ☐ 282 ☐ 283 ☐ 284 ☐ 285 ☐ 286 ☐ 287 ☐ 288 ☐ 289 ☐ 290 ☐ 291 ☐ 292 ☐ 293 ☐ 294 ☐ 295 ☐ 296 ☐ 297 ☐ 298 ☐ 299 ☐ 300 ☐ 301 ☐ 302 ☐ 303 ☐ 304 ☐ 305 ☐ 306 ☐ 307 ☐ 308 ☐ 309 ☐ 310 ☐ 311 ☐ 312 ☐ 313 ☐ 314 ☐ 315 ☐ 316 ☐ 317 ☐ 318 ☐ 319 ☐ 320 ☐ 321 ☐ 322 ☐ 323 ☐ 324 ☐ 325 ☐ 326 ☐ 327 ☐ 328 ☐ 329 ☐ 330 ☐ 331 ☐ 332 ☐ 333 ☐ 334 ☐ 335 ☐ 336 ☐ 337 ☐ 338 ☐ 339 ☐ 340 ☐ 341 ☐ 342 ☐ 343 ☐ 344 ☐ 345 ☐ 346 ☐ 347 ☐ 348 ☐ 349 ☐ 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☐ 517 ☐ 518 ☐ 519 ☐ 520 ☐ 521 ☐ 522 ☐ 523 ☐ 524 ☐ 525 ☐ 526 ☐ 527 ☐ 528 ☐ 529 ☐ 530 ☐ 531 ☐ 532 ☐ 533 ☐ 534 ☐ 535 ☐ 536 ☐ 537 ☐ 538 ☐ 539 ☐ 540 ☐ 541 ☐ 542 ☐ 543 ☐ 544 ☐ 545 ☐ 546 ☐ 547 ☐ 548 ☐ 549 ☐ 550 ☐ 551 ☐ 552 ☐ 553 ☐ 554 ☐ 555 ☐ 556 ☐ 557 ☐ 558 ☐ 559 ☐ 560 ☐ 561 ☐ 562 ☐ 563 ☐ 564 ☐ 565 ☐ 566 ☐ 567 ☐ 568 ☐ 569 ☐ 570 ☐ 571 ☐ 572 ☐ 573 ☐ 574 ☐ 575 ☐ 576 ☐ 577 ☐ 578 ☐ 579 ☐ 580 ☐ 581 ☐ 582 ☐ 583 ☐ 584 ☐ 585 ☐ 586 ☐ 587 ☐ 588 ☐ 589 ☐ 590 ☐ 591 ☐ 592 ☐ 593 ☐ 594 ☐ 595 ☐ 596 ☐ 597 ☐ 598 ☐ 599 ☐ 600 ☐ 601 ☐ 602 ☐ 603 ☐ 604 ☐ 605 ☐ 606 ☐ 607 ☐ 608 ☐ 609 ☐ 610 ☐ 611 ☐ 612 ☐ 613 ☐ 614 ☐ 615 ☐ 616 ☐ 617 ☐ 618 ☐ 619 ☐ 620 ☐ 621 ☐ 622 ☐ 623 ☐ 624 ☐ 625 ☐ 626 ☐ 627 ☐ 628 ☐ 629 ☐ 630 ☐ 631 ☐ 632 ☐ 633 ☐ 634 ☐ 635 ☐ 636 ☐ 637 ☐ 638 ☐ 639 ☐ 640 ☐ 641 ☐ 642 ☐ 643 ☐ 644 ☐ 645 ☐ 646 ☐ 647 ☐ 648 ☐ 649 ☐ 650 ☐ 651 ☐ 652 ☐ 653 ☐ 654 ☐ 655 ☐ 656 ☐ 657 ☐ 658 ☐ 659 ☐ 660 ☐ 661 ☐ 662 ☐ 663 ☐ 664 ☐ 665 ☐ 666 ☐ 667 ☐ 668 ☐ 669 ☐ 670 ☐ 671 ☐ 672 ☐ 673 ☐ 674 ☐ 675 ☐ 676 ☐ 677 ☐ 678 ☐ 679 ☐ 680 ☐ 681 ☐ 682 ☐ 683 ☐ 684 ☐ 685 ☐ 686 ☐ 687 ☐ 688 ☐ 689 ☐ 690 ☐ 691 ☐ 692 ☐ 693 ☐ 694 ☐ 695 ☐ 696 ☐ 697 ☐ 698 ☐ 699 ☐ 700 ☐ 701 ☐ 702 ☐ 703 ☐ 704 ☐ 705 ☐ 706 ☐ 707 ☐ 708 ☐ 709 ☐ 710 ☐ 711 ☐ 712 ☐ 713 ☐ 714 ☐ 715 ☐ 716 ☐ 717 ☐ 718 ☐ 719 ☐ 720 ☐ 721 ☐ 722 ☐ 723 ☐ 724 ☐ 725 ☐ 726 ☐ 727 ☐ 728 ☐ 729 ☐ 730 ☐ 731 ☐ 732 ☐ 733 ☐ 734 ☐ 735 ☐ 736 ☐ 737 ☐ 738 ☐ 739 ☐ 740 ☐ 741 ☐ 742 ☐ 743 ☐ 744 ☐ 745 ☐ 746 ☐ 747 ☐ 748 ☐ 749 ☐ 750 ☐ 751 ☐ 752 ☐ 753 ☐ 754 ☐ 755 ☐ 756 ☐ 757 ☐ 758 ☐ 759 ☐ 760 ☐ 761 ☐ 762 ☐ 763 ☐ 764 ☐ 765 ☐ 766 ☐ 767 ☐ 768 ☐ 769 ☐ 770 ☐ 771 ☐ 772 ☐ 773 ☐ 774 ☐ 775 ☐ 776 ☐ 777 ☐ 778 ☐ 779 ☐ 780 ☐ 781 ☐ 782 ☐ 783 ☐ 784 ☐ 785 ☐ 786 ☐ 787 ☐ 788 ☐ 789 ☐ 790 ☐ 791 ☐ 792 ☐ 793 ☐ 794 ☐ 795 ☐ 796 ☐ 797 ☐ 798 ☐ 799 ☐ 800 ☐ 801 ☐ 802 ☐ 803 ☐ 804 ☐ 805 ☐ 806 ☐ 807 ☐ 808 ☐ 809 ☐ 810 ☐ 811 ☐ 812 ☐ 813 ☐ 814 ☐ 815 ☐ 816 ☐ 817 ☐ 818 ☐ 819 ☐ 820 ☐ 821 ☐ 822 ☐ 823 ☐ 824 ☐ 825 ☐ 826 ☐ 827 ☐ 828 ☐ 829 ☐ 830 ☐ 831 ☐ 832 ☐ 833 ☐ 834 ☐ 835 ☐ 836 ☐ 837 ☐ 838 ☐ 839 ☐ 840 ☐ 841 ☐ 842 ☐ 843 ☐ 844 ☐ 845 ☐ 846 ☐ 847 ☐ 848 ☐ 849 ☐ 850 ☐ 851 ☐ 852 ☐ 853 ☐ 854 ☐ 855 ☐ 856 ☐ 857 ☐ 858 ☐ 859 ☐ 860 ☐ 861 ☐ 862 ☐ 863 ☐ 864 ☐ 865 ☐ 866 ☐ 867 ☐ 868 ☐ 869 ☐ 870 ☐ 871 ☐ 872 ☐ 873 ☐ 874 ☐ 875 ☐ 876 ☐ 877 ☐ 878 ☐ 879 ☐ 880 ☐ 881 ☐ 882 ☐ 883 ☐ 884 ☐ 885 ☐ 886 ☐ 887 ☐ 888 ☐ 889 ☐ 890 ☐ 891 ☐ 892 ☐ 893 ☐ 894 ☐ 895 ☐ 896 ☐ 897 ☐ 898 ☐ 899 ☐ 900 ☐ 901 ☐ 902 ☐ 903 ☐ 904 ☐ 905 ☐ 906 ☐ 907 ☐ 908 ☐ 909 ☐ 910

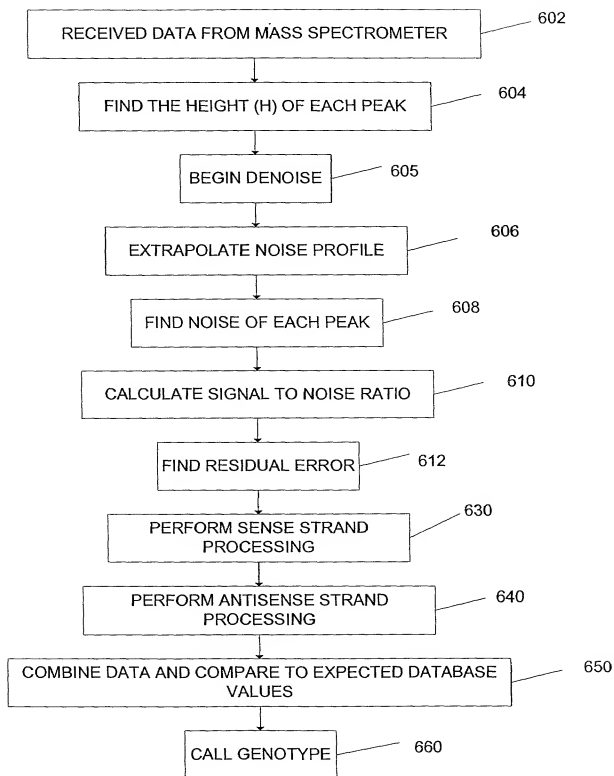


FIGURE 23

09687483.101300

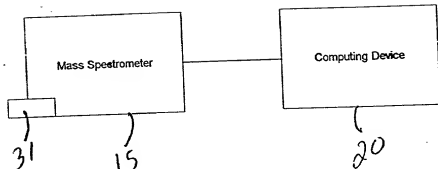
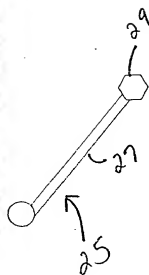


FIGURE 24

10  
↓

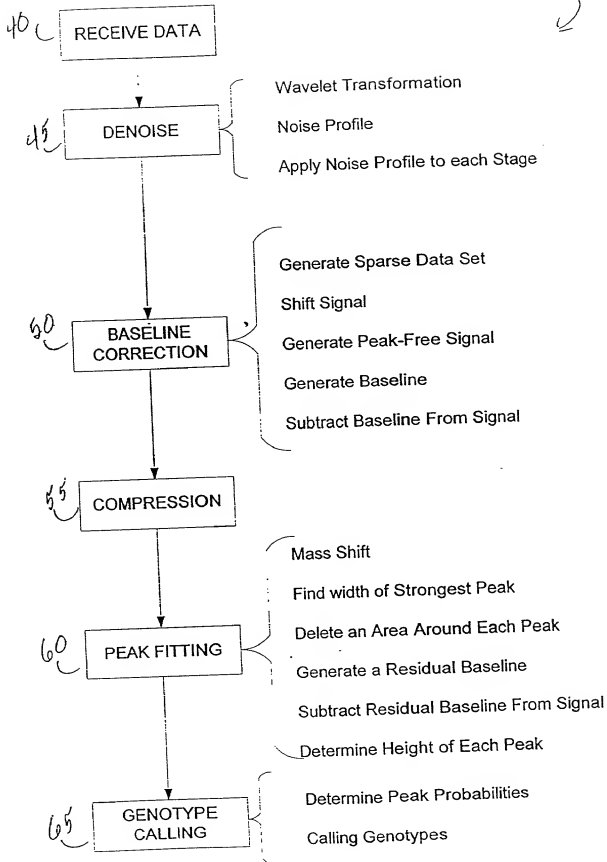


FIGURE 25

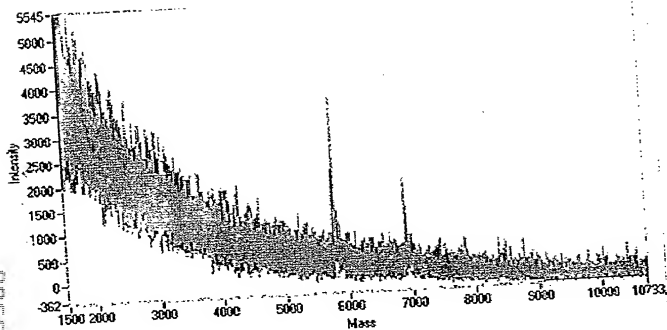


FIGURE 26

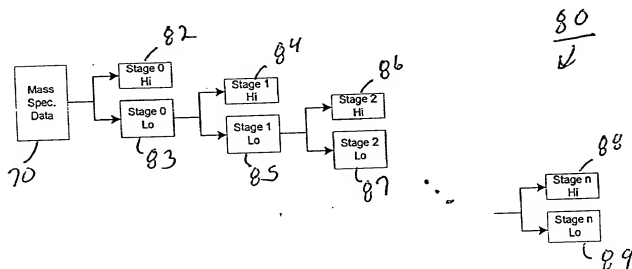


FIGURE 27

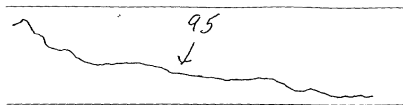
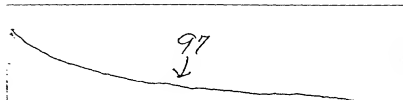


FIGURE 28



Exp fitting  
 $a_0 + a_1 \exp(a_2 m)$

FIGURE 29

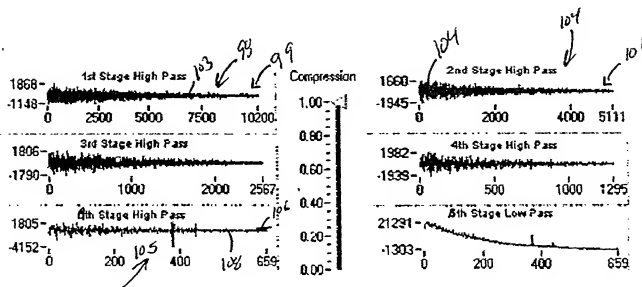


FIGURE 30

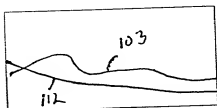
006701-28+28960

Stage 0 - Hi



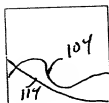
Threshold 0=4XNoiseProfile

Stage 1 - Hi



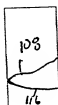
Threshold 1=2XNoiseProfile

Stage 2 - Hi



Threshold 2=1XNoiseProfile

Stage n - Hi



Threshold  $n=(1/2^{n-2})XNoiseProfile$

Stage n - Lo

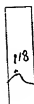


FIGURE 31

120  
✓

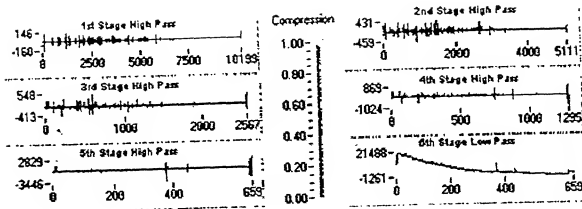


FIGURE 32

$$\text{Signal}(t) = \frac{(\text{Start } 0(t) + \text{Start } 1(t) + \text{Start } 2(t) \dots + \text{Start } 23(t))}{24}$$

FIGURE 33

SHIFT SIGNAL TO ACCOUNT FOR  
VARIATIONS DUE TO STARTING POINT

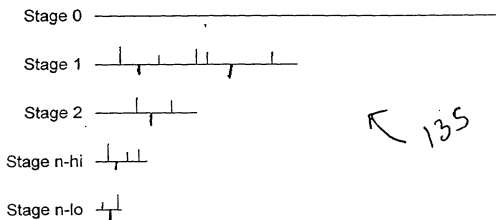


FIGURE 34

002101-88428960

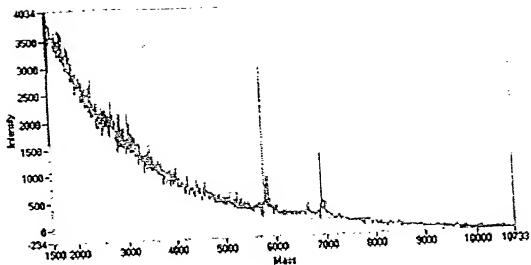


FIGURE 35

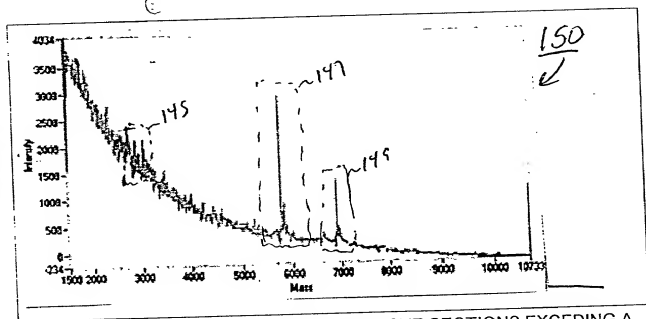


FIG. 13 - TAKE A MOVING AVERAGE, REMOVE SECTIONS EXCEEDING A THRESHOLD

FIGURE 36

00667483-103300

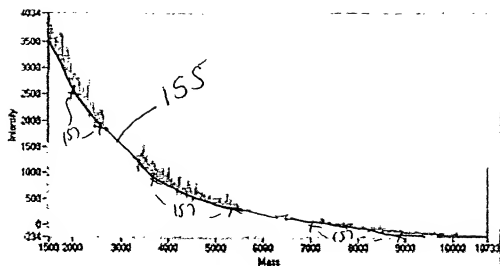


FIGURE 37

FIND MINIMA IN REMAINING SIGNALS AND CONNECT TO FORM A PEAK FREE SIGNAL

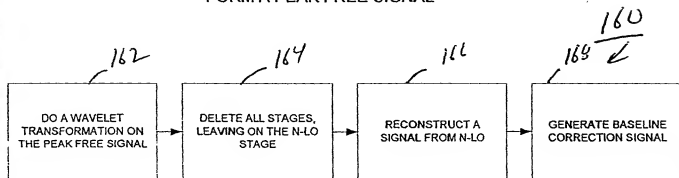


FIGURE 38

GENERATE BASELINE CORRECTION

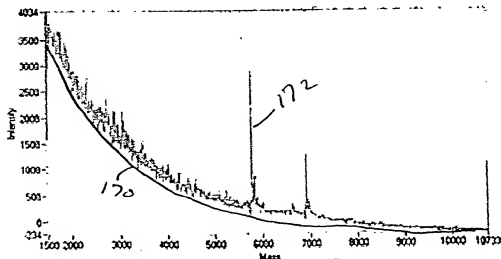


FIGURE 39

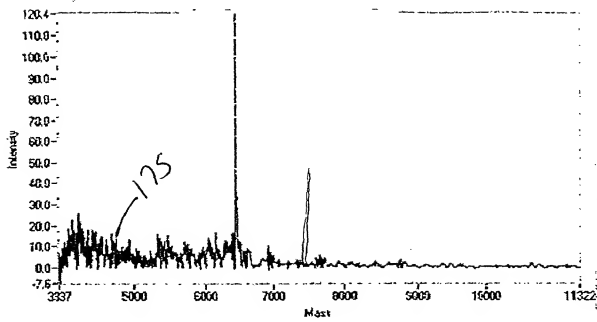


FIGURE 40

00587483-101300

NON-0 COEFFICIENTS	VALUE	INTERMEDIATE	RELATIVE
100	25	100.025	100.025
150	220	150.220	50.220
500	.1	500.0001	350.0001
10,050	800	10,050.8	9550.8
10,075	890	10,075.89	25.89
11,125	910	11,125.91	150.91
12,100	1000 (MAX)	12,100.99999	975.99999
13,250	940	13,250.94	1150.94

B<sub>2</sub>

184

185

186

190

180  
2

195

183

FIGURE 41

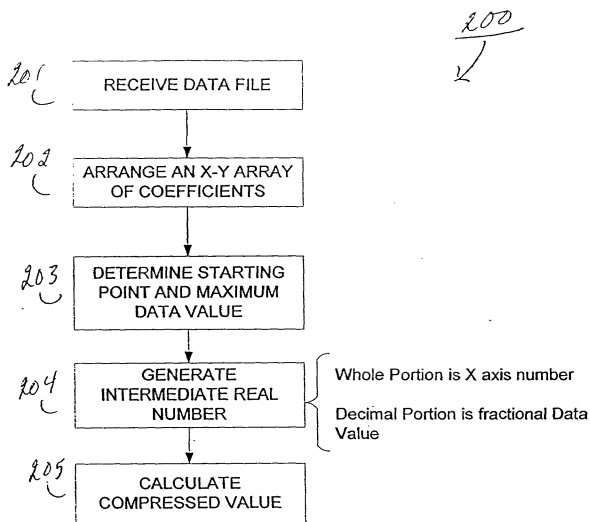


FIGURE 42

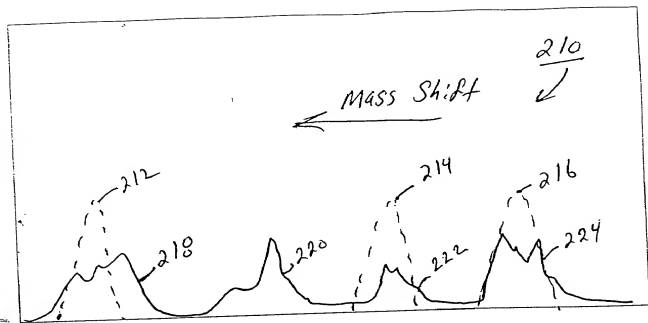


FIGURE 43

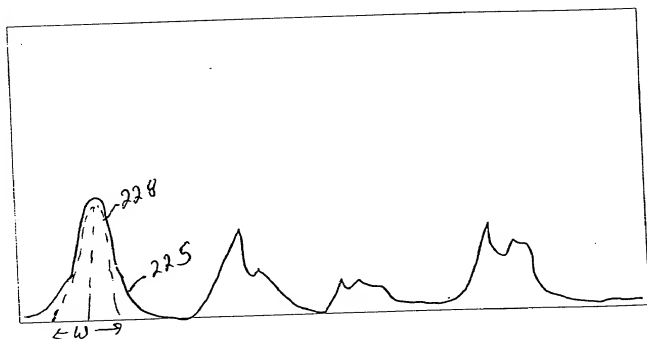


FIGURE 44

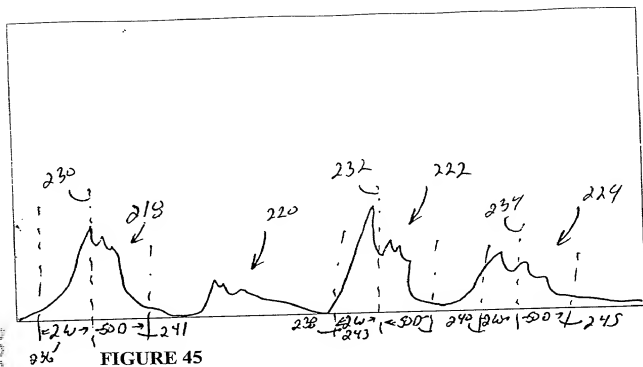


FIGURE 45

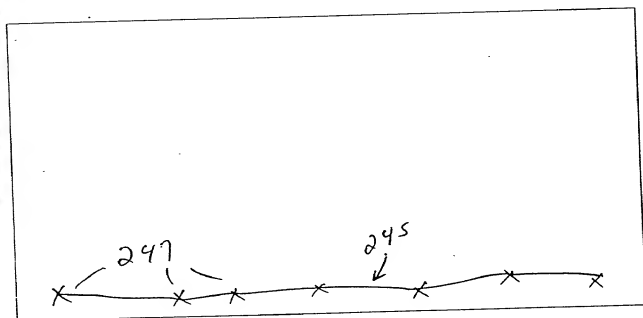


FIGURE 46

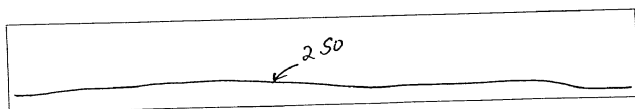


FIGURE 47

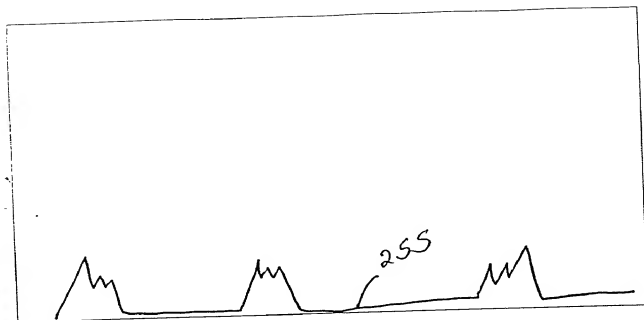


FIGURE 48

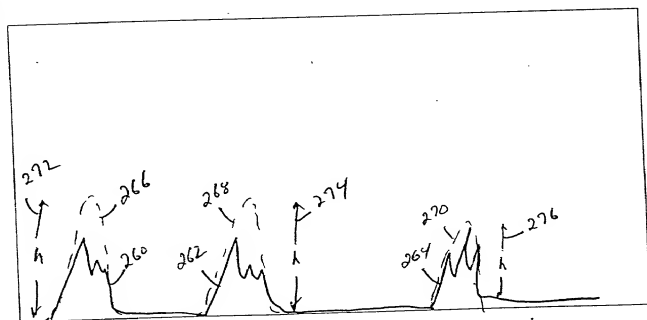


FIGURE 49

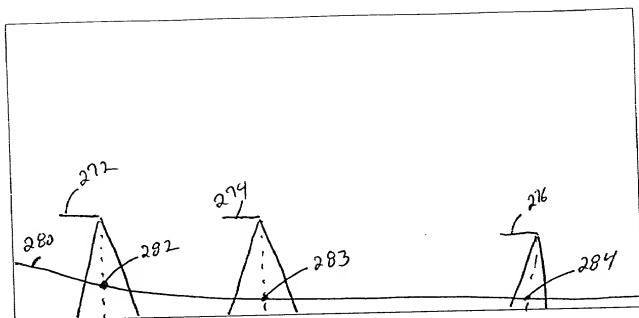


FIGURE 50

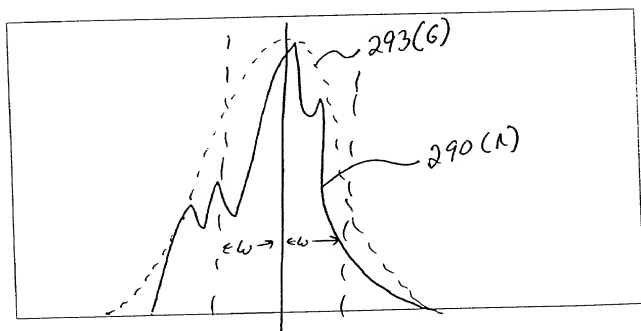


FIGURE 51

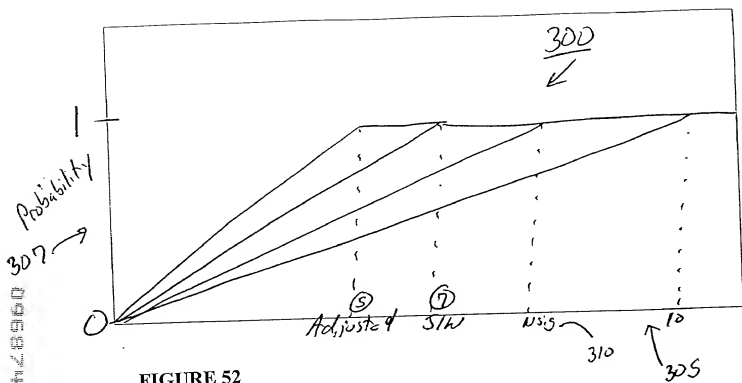


FIGURE 52

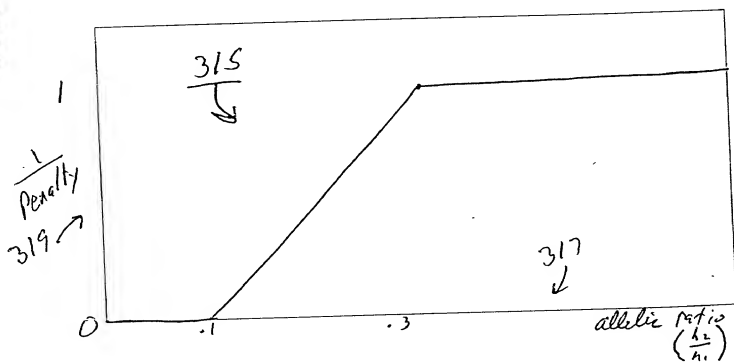


FIGURE 53

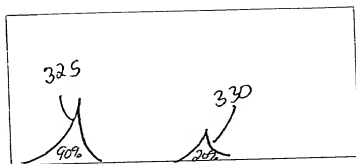


FIGURE 54

329

PROBABILITY OF GG EXISTING:  
 $P(GG) = P(G) * P(1-C)$   
 $= 90\% * (100\% - 20\%)$   
 $= 90\% * 80\%$   
 $= 72\%$

331

PROBABILITY OF CC EXISTING:  
 $P(CC) = P(C) * P(1-G)$   
 $= 20\% * (100\% - 90\%)$   
 $= 20\% * 10\%$   
 $= 2\%$

333

PROBABILITY OF GC EXISTING:  
 $P(GC) = P(G) * P(C)$   
 $= 90\% * 20\%$   
 $= 18\%$

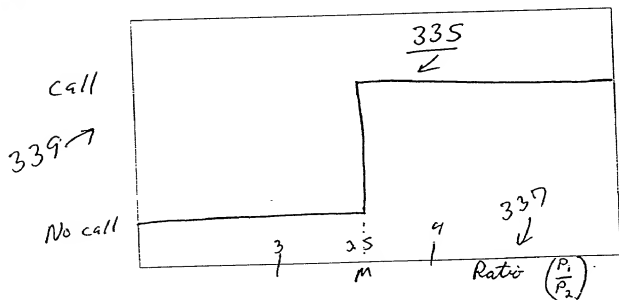


FIGURE 55

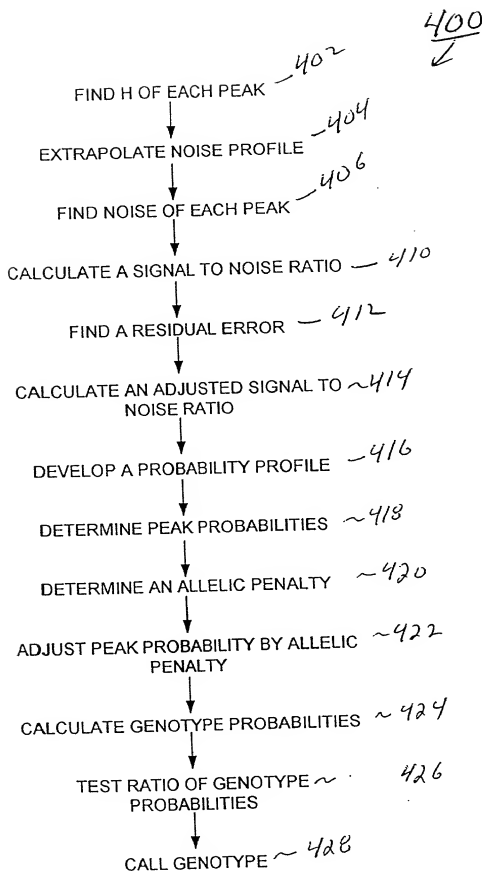


FIGURE 56

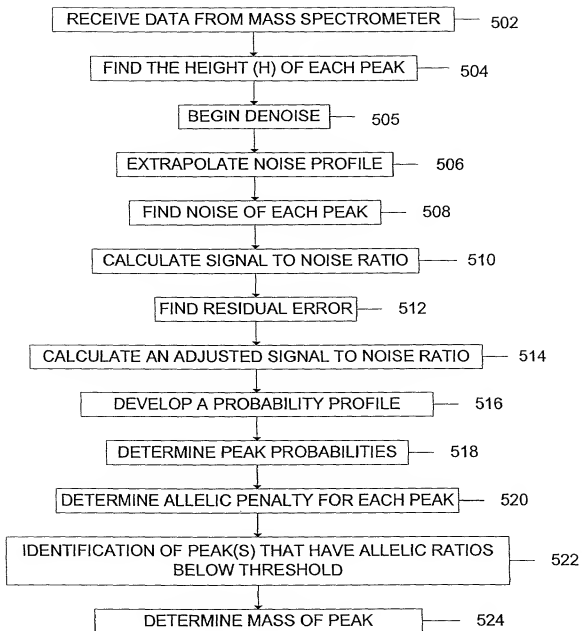


FIGURE 57

09687483-101300

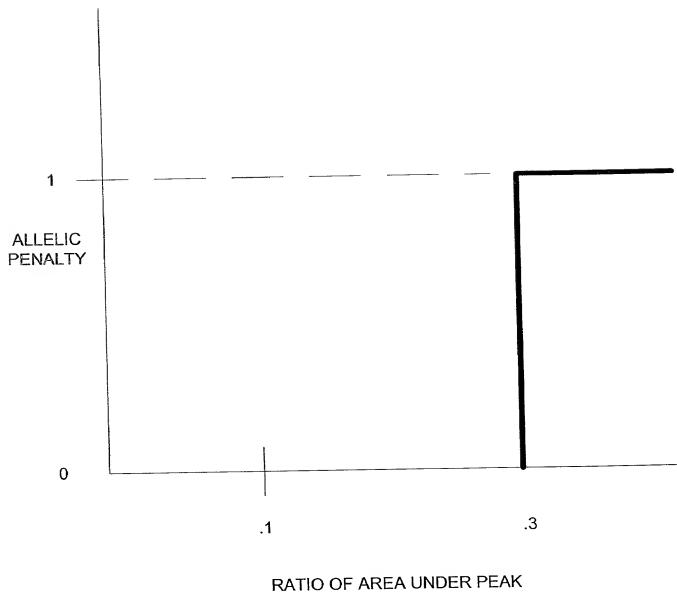


FIGURE 58

[illegible]

<120> METHODS FOR GENERATING DATABASES AND DATABASES FOR IDENTIFYING  
POLYMORPHIC GENETIC MARKERS

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&lt;150&gt; 60/217,658

&lt;150&gt; 60/159.176

<150> 60/217.251

<150> 09/663,968

<151> 2000-09-19

<170> FastSEO for Windows Version 4.0

<211> 361

<212> DNA

<213> Homo Sapien

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aggtccgat	gaagctccca	gaatgccaca	ggctgtcccc	ccgtggccc	ctgcaccage	180
agctctacta	cggcgggccc	ctgcagcacg	ccccctctgg	ccctgtcat	ctctctgcc	240
ttccaccaaa	acctaccagg	cgcagtcagg	ttccgctgt	ggctctctgc	attctgggac	300
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a						361

<210> 2

<211> 44

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$ 

<223> Oligonucleotide Primer

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 $\langle 210 \rangle$  3

$\langle 211 \rangle$  42

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$ 

<223> Oligonucleotide Primer

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<210> 4

<211> 17

06664-3960

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<220>  
<223> Probe

```
<400> 5
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```

<220>  
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<400> 7  
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<213> Homo Sapien
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gagcagctga gcgcgcactg tcatgcgcta atggcgggct gcatccagga ggcccgtag 120
cgatggaact cgcactttgt caccgagaca ccactggagg g 161
```

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<211> 43
<212> DNA
<213> Artificial Sequence
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<220>  
<223> Oligonucleotide Primer

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```
<210> 10
<211> 44
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<213> Artificial Sequence
```

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<211> 15		
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<211> 205		
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<213> Homo Sapien		
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caggtgcagt tccaaggaag cctttgagaa agggctctgc ttgagttgta gaaagaaccg		120
ctgcaacaat ctgggctatg agatcaataa agtcagagcc aaaagaagca gcaaaatgta		180
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<220>		

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 <210> 17  
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 <211> 20  
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 <220>  
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 <210> 19  
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 <212> DNA  
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 caatctgggc tatgatgatca 20  
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 <223> Probe

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 <223> Oligonucleotide primer  
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 <223> Probe  
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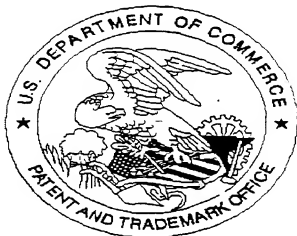
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